MOLECULAR MECHANISMS IN HUMAN HEPATOCELLULAR CARCINOMA

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Abstract

Hepatocellular carcinoma (HCC) is one of the commonest cancers worldwide. There is, however, a marked geographical variation in incidence and it has been suggested that the pathogenesis may vary in different parts of the world.

A retrospective analysis of 110 HCC patients was initially undertaken which confirmed that only 29% of British patients had markers of hepatitis B infection, suggesting a possible role for other environmental agents in the pathogenesis, and that 80% of patients had underlying cirrhosis.

The nature of the strong relationship between HCC and cirrhosis has not been established but it has been postulated that increased hepatocyte turnover in the cirrhotic liver may predispose to DNA damage by environmental mutagens. Cell proliferation is required to express the strongly promutagenic DNA base lesion O^6-methylguanine, produced by alkylating agents, as a mutation. O^6-methylguanine is repaired by the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (O^6-MT). A microassay was developed which could reliably measure O^6-MT levels in liver biopsy samples. Using this approach O^6-MT levels were found to be significantly lower in cirrhotic liver when compared to non-cirrhotic and normal liver tissue. No correlation was found between lymphocyte and liver levels from individual patients with liver disease indicating that the deficiency in DNA repair is disease- and tissue-specific.

Three polyclonal antibodies were subsequently raised to O^6-MT peptides and characterised by immunoblotting in an attempt to establish the tissue distribution of the enzyme in liver. Although none of the antisera were able to detect O^6-MT in tissue sections they were used to analyse structural differences in the enzyme between cirrhotic and non-cirrhotic liver using SDS-PAGE followed by immunoblotting and fluorography. A band of M_r 24,000, representing native enzyme, was visualised by fluorography in all liver extracts. Densitometry of these bands correlated with the enzyme activity determined by the direct enzyme assay, validating the assay findings. Other small molecular weight bands were seen in all liver extracts and comparison with immunoblots suggested that these bands represent C-terminal truncated enzyme. The spectrum of smaller molecular weight enzyme forms was similar in cirrhotic and non-cirrhotic liver. It was, thus, concluded that although O^6-MT levels were lower in cirrhosis this was not accounted for by structural differences in the enzyme.

DNA mutations (G to A) produced by the failure to repair O^6-methylguanine are known to activate oncogenes and tumour suppressor genes such as p53. However only 5/55 (9%) of HCC expressed mutant p53. Other factors potentially involved in hepatocarcinogenesis include the growth factor TGF-α and a growth factor receptor encoded by the c-*erb* B-2 proto-oncogene. Expression of TGF-α and the c-*erb* B-2 oncoprotein were seen in 8/28 (28%) and 2/26 (8%) of HCC respectively, findings which differ from those observed in HCC from the Far East.

Deficient DNA repair by O^6-MT provides one possible reason why cirrhosis is an important risk factor for the development of HCC. However, failure to repair O^6-methylguanine does not result in mutations within the p53 gene in British HCC. Furthermore, the finding of low expression of mutant p53, TGF-α and the c-*erb* B-2 oncoprotein in HCC from Britain compared to HCC from the Far East and Africa suggests geographical differences in the molecular mechanisms involved in hepatocarcinogenesis between areas of high and low HCC prevalence.

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Collier J D, Guo K, Gullick W, Bassendine M F, Burt A D. Expression of transforming growth factor alpha (TGF-α) in human hepatocellular carcinoma. Liver 1993; **13**: 151-155.

Collier J D, Curless R, Bassendine M F, James O F W. Clinical features and prognosis of hepatocellular carcinoma in Britain with respect to advancing age. Age and ageing 1993 (in press).

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List of abbreviations

aa	amino acid
α-FP	alpha-fetoprotein
BSA	Bovine serum albumin
CLD	Chronic liver disease
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblast growth factor
[^{3}H-Me] O^6-MT	Tritiated methylated native O^6-MT
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HPLC	High performance liquid chromatography
IGF	Insulin-like growth factor
O^6-MT	O^6-methylguanine-DNA methyltransferase
PBS	Phosphate buffered saline, pH7.4
PDGF	Platelet derived growth factor
rO^6-MT	Recombinant O^6-MT
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline, pH 7.6
TGF-α	Transforming growth factor alpha

Contents

Chapter 1

Introduction

1.1 Hepatocellular carcinoma

1.1.1 Background

Primary liver tumours can be classified histologically as either epithelial or mesenchymal. Epithelial tumours include hepatocellular carcinomas (HCC), intrahepatic cholangiocarcinomas and hepatoblastomas whereas mesenchymal tumours, accounting for only 1% of primary liver malignancies, include angiosarcomas, epithelial haemangioendotheliomas and undifferentiated sarcomas.

Hepatocellular carcinoma (HCC) is a malignant tumour of hepatocytes and accounts for 80% of primary liver tumours. Histologically HCC usually has either a trabecular or a pseudoglandular (adenoid) architecture but other rarer histological subtypes are also recognised such as fibrolamellar tumours which occur at a younger age and carry a better prognosis. A clear cell variant of HCC is also described and there is some evidence that this also carries a more favourable prognosis.

1.1.2 Incidence

Hepatocellular carcinoma remains one of the commonest cancers worldwide with 251,000 new cases reported each year (Parkin et al, 1988). It accounts for 4% of all tumours and is the seventh commonest cancer worldwide. Recent studies have also suggested that the incidence is increasing in Northern Europe, where there has been a 3 fold increase over the last 20 years, and in Japan (Bosch and Munoz, 1991). An important feature of HCC is the remarkable geographical variation in incidence. Age-standardised yearly incidence rates vary from < 4 per 100,000 in the UK to 50 per 100,000 in China and Hong Kong, and >100 per 100,000 in Mozambique (Cook-Mozaffari et al, 1984). Thus 45% of new cases occur in China, 10% in Africa and 6% in Europe.

1.1.3 Aetiology

The striking worldwide variation in incidence suggests an important role for environmental factors in the aetiology of HCC (reviewed by Bassendine, 1987). Factors implicated include viruses and chemicals; there is evidence of geographical variation in exposure to these agents amongst HCC patients. Epidemiological evidence also indicates that the aetiology of HCC is probably multifactorial with individual exposure occuring to more than one potential carcinogen (Bassendine, 1987).

1.1.3.1 Age and sex

The incidence of HCC increases with age; this feature is in common with other human malignancies (Goodwin et al, 1986). However, there are also some geographical differences in that the peak age of onset is earlier, at 37 years, in South Africa (Kew and Geddes, 1982) compared to 65 years in Japan and Northern Europe (The Liver Cancer Study Group Of Japan, 1987; Dunk et al, 1988).

HCC occurs more frequently in men with a male to female ratio of up to 8:1 in South East Asia (Lai et

al, 1981) and a ratio of 3:1 in Northern Europe (Ihde et al, 1974). In the West this male predominance is accounted for by the presence of HCC complicating cirrhosis, non-cirrhotic HCC having a 1:1 male to female ratio (Melia et al, 1984) whereas in Japan the male predominance exists for both cirrhotics and non-cirrhotics (Okuda et al, 1980). Although the relationship between male sex and HCC is not fully resolved the finding of a raised oestrone to testosterone ratio in male cirrhotic patients with HCC compared to those with cirrhosis alone has led to the hypothesis that hyperoestrogenaemia is a risk factor for HCC (Nagasue et al, 1985). Oestrogens increase the number of liver tumours in animal models of hepatocarcinogenesis (Wanless and Medline, 1982) and higher levels of oestrogen receptors have been reported in tumour when compared to surrounding normal liver (Sainsbury et al, 1984). Long term use of the oestrogen-containing contraceptive pill is associated with the development of HCC (Goodman and Ishak, 1982; Neuberger et al, 1986); although this relationship is controversal.

1.1.3.2 Viruses

Hepatitis B

There is strong epidemiological evidence supporting an aetiological role for chronic hepatitis B infection in HCC, with a worldwide correlation between the incidence of HCC and prevalence of the HBsAg carrier-state (Szmuness,1978). This relationship has been confirmed by case-controlled studies showing a twenty-fold increased relative risk of developing HCC in association with chronic HBV infection (Yeh et al,1985). Prospective studies of HBsAg carriers from high and low incidence areas also show a high relative risk (50-100 fold) of developing HCC (Beasley et al, 1981; Hall et al, 1985). HBV-related cirrhosis also carries a higher risk of malignancy than other types of cirrhosis (reviewed by Slagle et al, 1992). However, the mechanisms involved in hepatitis B-related hepatocarcinogenesis are still unresolved. Integrated HBV DNA sequences have been detected in chromosomal DNA in tumour liver but these occur randomly (Himo et al, 1984; Shafritz and Kew, 1981) and it is therefore unclear how these viral sequences can uniformally disrupt normal hepatocyte function leading to tumour production.

Hepatitis C

Hepatitis C (HCV) is an RNA virus which is now recognised as a major cause of non-A non-B hepatitis (Choo, 1990) and a risk factor for HCC. The prevalence of HCV infection in the normal population varies geographically, from 0.18% in Great Britain (Brind et al, 1990) to 0.5% in Italy (Sirchia et al, 1989) and 3% in Japan (Tanaka et al, 1991). Antibodies to HCV have been detected in up to 80% of patients with HCC. However, there is a marked geographical variation with serological evidence of HCV infection in HCC patients varying from 12% in China (Lee et al, 1992) and 29% in South Africa (Kew, 1990) to 51% in Japan (Tanaka et al, 1991) and 65% in Italy (Colombo et al, 1989) . There is as yet no data on the prevalence of hepatitis C infection in HCC in Britain. A recent study has looked for evidence of active viral replication in HCC and identified HCV RNA in the serum of 35 % of Swiss patients with HCC (Garson et al, 1992). HCV RNA has also been detected in malignant hepatocytes (Chou et al,1991). These findings together with the studies using HCV serology are very suggestive of a role for HCV in the pathogenesis of HCC.

1.1.3.3 Chemicals

A wide variety of chemicals, including *N*-nitroso compounds and chlorinated hydrocarbons, are known to produce liver cell tumours in experimental animals (reviewed by Grasso, 1987). Although man is exposed to many of these compounds, which are ubiquitous environmental chemicals, a link between such agents and HCC has not been fully explored. The potential role for *N*-nitroso compounds in human hepatocarcinogenesis is discussed later in this chapter. It is, however, known that exposure to other chemicals such as organic solvents results in a 2 fold increased risk of developing HCC (Hardell et al, 1984). The link between HCC in China and exposure to drinking water obtained from stagnant ponds has led to speculation that exposure to organochloride pesticides may also be involved in hepatocarcinogenesis (Delong, 1979).

Aflatoxin B_1 is produced by certain strains of Aspergillus flavus, which contaminates cereals and nuts during storage. It is a recognised carcinogen in animal models, inducing hepatocellular carcinoma in rats and primates (Busby and Wogan, 1984). In man, epidemiological evidence has linked exposure to aflatoxin with HCC in Africa (Linsell and Peers, 1977) , Far East (Yaobin et al, 1983), and South Africa (Kew et al, 1993). It is accepted that exposure to aflatoxin is low in Japan and Europe but this is based on the lack of exposure to stored foods known to be contaminated by aspergillus and has not been established epidemiologically. Aflatoxin has been found in the serum and liver of patients with HCC (Onyemelukwe et al, 1982) and DNA adducts, which aflatoxin forms with guanine in DNA (Muench et al 1983), have also been identified in liver tumour tissue (Zhang et al, 1991). However, a direct causal role for aflatoxin in man is unproven and any association between aflatoxin and other risk factors such as hepatitis B infection, which is high in areas of high aflatoxin exposure, remains to be characterised.

1.1.4 Clinical features

HCC is a tumour with a poor prognosis (reviewed by Johnson, 1987); the overall median survival is 4 months. Early diagnosis is difficult as symptoms are usually non-specific and most tumours present at a late stage. The recognition that the majority of patients with HCC have underlying cirrhosis has led to screening of cirrhotic patients by serum alpha fetoprotein estimations and hepatic ultrasound scanning (Okuda, 1986). This has resulted in the detection of early tumours in Japan which are amenable to surgery with some improvement in prognosis. These results, however, have not been reproduced in Europe (Colombo et al, 1991). The only curative treatment for HCC is surgical resection or liver transplantation but few patients are suitable candidates because of advanced tumour stage at diagnosis (reviewed Guest and Blumgart, 1987; Poulson et al, 1987). There have been few advances in chemotherapy for HCC which essentially remains palliative (reviewed Dunk and Thomas, 1988).

Improvement in the prognosis of HCC, which thus remains a common, rapidly growing, chemotherapeutic resistant tumour, is likely to depend on an improvement in our understanding of

the mechanisms involved in hepatocarcinogenesis, such as those involved in the malignant transformation of cirrhotic liver and the factors contributing to the rapid growth of the tumour.

1.2 Cirrhosis as a preneoplastic lesion

A close association exists between cirrhosis and HCC. This is based on the finding of a high prevalence of cirrhosis in patients with HCC and on the observation of an increased risk of patients with cirrhosis developing HCC. In 60-90% of all cases of HCC, cirrhosis is present in the adjacent liver and unlike other postulated aetiological factors, there is less geographical variation in the prevalence of cirrhosis in HCC patients (Table 1.2). Cirrhosis is thus an important worldwide risk factor for the development of HCC.

Table 1.2: Worldwide incidence of cirrhosis in patients with HCC.

Country	Incidence of HCC	% of HCC patients with cirrhosis	Reference
South Africa	High	61	Kew et al, 1992
Japan	Intermediate	80	Nagasue et al,1984
Italy	Intermediate	95	Villa et al, 1988
Britain	Low	80	Dunk et al, 1988

Cirrhosis is a pathological diagnosis made on the basis of loss of the normal liver architecture with the formation of regenerating nodules and fibrosis. It occurs as the result of chronic liver cell damage such as occurs through hepatitis B infection and immune mediated damage in chronic autoimmune liver disease. Cirrhosis can be subdivided, according to its aetiology, as follows: (i) viral; hepatitis B and C, (ii) toxins; alcohol, (iii) autoimmune; primary biliary cirrhosis and chronic active hepatitis, and (iv) metabolic; haemochromatosis, Wilsons disease and alpha 1 antitrypsin deficiency. In some cases the aetiology is not apparant (cryptogenic cirrhosis). The aetiology of cirrhosis also varies geographically; hepatitis B being the commonest cause in the Far East and alcohol the major aetiological factor in Northern Europe.

The risk of cirrhotic patients developing HCC depends on it's aetiology; hepatitis B-related cirrhosis and haemochromatosis carry a much greater risk than primary biliary cirrhosis (Bassendine, 1987). Despite the strong association between cirrhosis and HCC the nature of the relationship between cirrhosis and HCC has not been established. One hypothesis is that the raised rate of DNA synthesis that accompanies the formation of hyperplastic nodules in cirrhosis could act as a promoter for cells already initiated by exposure to a primary carcinogen, viral or chemical. The development of methodology to measure DNA synthesis, *in vitro*, in liver biopsies using uptake of

bromodeoxyuridine (BrDU), a thymidine analogue, taken up by S phase cells and detected using anti-BrdU antibodies, has allowed indirect assessment of cell proliferation in cirrhotic tissue (Gratzner, 1982). Using this technique, DNA synthesis was found to be higher in cirrhotic tissue adjacent to tumour involved tissue than in cirrhotic tissue taken from patients without HCC (Tarao et al, 1989) and a prospective study of cirrhotic patients has shown an increased risk of developing HCC in those patients with higher levels of DNA synthesis (Tarao et al, 1992). Cells within cirrhotic tissue are therefore arguably at increased risk of damage from environmental carcinogens, both viral and chemical.

1.3 Chemical carcinogenesis

Tumorigenesis in humans in a complex multistep process (reviewed by Weinberg, 1989; Fearson and Vogelstein, 1990; Bishop, 1987). It can only be studied indirectly in man and most of our understanding of the molecular mechanisms involved comes from studies in animals. Experimental carcinogenesis has traditionally been divided into three steps; initiation, promotion and progression (Archer, 1987). Although this is an oversimplification of a complex pattern of events, it does serve as a useful frame work when examining elements involved in chemical carcinogenesis. Initiation is an irreversible process by which chemical carcinogens or ionizing radiation causes permanent DNA damage in target cells. Chemical damage can be mediated through exposure to environmental carcinogens or to endogenous carcinogens (Ames, 1990). Epidemiological evidence has linked occupational exposure to various chemicals with the development of human tumours (reviewed Archer, 1987). Thus, exposure to polycyclic hydrocarbons is linked to scrotal cancer and napthylamine exposure with bladder cancer. The liver is also susceptible to damage by chemical carcinogens as exemplified by the strong link between vinyl chloride exposure and the development of hepatic angiosarcoma (Berk, 1976). The ultimate effect of chemically-induced DNA damage is dependent on the presence of DNA repair mechanisms. If damage is not repaired prior to DNA replication then it will become fixed in the daughter cells . An important type of damage appears to be activation of oncogenes and/or alteration of the function of tumour suppressor genes by point mutations. Oncogenes, such as the activated *ras* genes, are mutated forms of normal cellular genes, proto-oncogenes, which are normally involved in regulating cell proliferation and differentiation (Bishop, 1987). Thus, oncogene activation through mutation leads to loss of normal control of cell growth. The pattern of oncogene activation appears to depend on tumour type and chemical carcinogen, with exposure to certain carcinogens being associated with specific mutations.

Promotion has been defined as the process whereby tumour formation is stimulated in tissues that have been exposed to an initiating agent (Archer, 1987). Cellular proliferation, or mitogenesis, is also an important factor in the process of carcinogenesis (Weinberg 1989) as exemplified by the use of tumour promoting agents (often chemicals), which increase cell turnover, in animal models of chemical carcinogenesis. Other factors that affect cell proliferation include oncogenes and growth factors. As well as causing mutations exposure to chemical carcinogens can also lead to cell death. This results in secondary cell proliferation and in this way chemical carcinogens can also act as

promotors (Ames 1990). Figure 1.3 illustrates the interplay between the exposure to chemical carcinogens, cell proliferation and tumour production.

1.4 Chemical hepatocarcinogenesis

1.4.1 Susceptibility of human liver to chemical carcinogens

Exposure to environmental factors, such as aflatoxin and hepatitis B, are known from epidemiological studies to be associated with the pathogenesis of human HCC. In Britain, however, exposure to aflatoxin is low and a significant number of patients with HCC have no markers of hepatitis B infection, suggesting that other environmental carcinogens may be important in the development of HCC.

The susceptibility of human liver to carcinogenic damage by environmental compounds depends on the following four factors (Craddock , 1986) :-

(1) The ability of the liver to metabolise the compounds to metabolites capable of reacting with DNA.

(2) The extent and specificity of DNA base damage produced.

(3) The presence of a repair mechanism capable of reversing DNA base damage prior to replication.

(4) The extent of DNA replication which will 'fix' any DNA damage as a mutation in newly divided cells.

The *N*-nitroso compounds are candidate carcinogens as they are ubiquitous environmental compounds which are primarily metabolised in the liver and cause DNA base damage which, if not repaired, results in G to A mutations which are known to activate oncogenes and tumour suppressor genes. These compounds are also recognised carcinogens in rodent models of hepatocarcinogenesis (Grasso et al , 1977).

1.4.2 *N*-nitroso compounds

Human exposure to *N*-nitroso compounds occurs in occupational and other environmental settings (reviewed by Bartsch and Montesano 1984). Occupational exposure occurs, for example, in the dry cleaning and petrol station service industries, and epidemiological evidence has shown that such workers are at risk of developing HCC (Stemhagen et al, 1983). Evidence for the carcinogenicity of *N*-nitroso compounds in food was recently strengthened by a case-controlled study from China that linked ingestion of *N*-nitroso compounds with oesophageal cancer (Cheng et al, 1992). Endogenous exposure to nitrosamines, produced in *vivo* from precursor amines, is thought by some (Bartsch and Montesano, 1984) to be more important than exogenous exposure from environmental sources. However, methods for quantifying the extent of nitrosamine exposure, both exogenous and

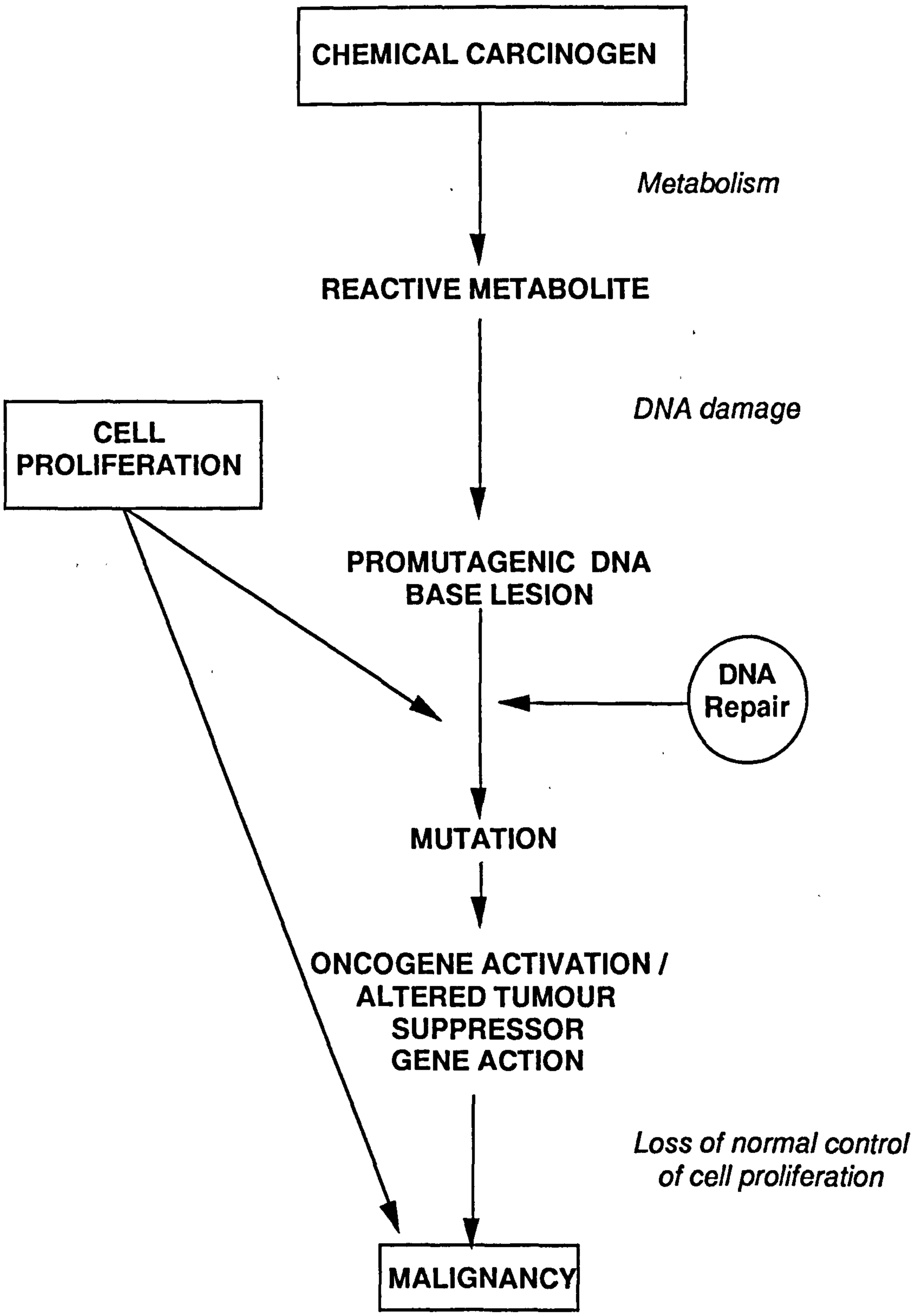


Figure 1.3: Hypothetical mechanism of chemical carcinogenesis.

endogenous, in humans are still being developed and so most of the evidence on the carcinogenic effect of nitrosamines on the liver relies on animal models (Fine, 1982).

N-nitroso compounds fall into two classes, the *N*-nitosamines and the *N*-nitrosamides. *N*-nitrosamides (e.g. methylnitrosurea) are characteristically unstable at physiological pH and rapidly decompose to reactive intermediates. In contrast, nitrosamines (e.g. dimethylnitrosurea) are relatively stable with their action on DNA being through active metabolites that are produced in cells by enzyme action (Montesano, 1981). Unlike many other chemical carcinogens, whose tumorgenicity in animals is species-specific, *N*-nitroso compounds produce liver tumours across the complete spectrum of animal species (Bogoviski et al, 1981). Carcinogens that are commonly used in rodent models of chemical hepatocarcinogenesis include the nitrosamines, dimethylnitrosourea and diethylnitrosourea, and the nitrosamide, methylnitrosourea (Farber and Cameron, 1980). *N*-nitroso compounds are alkylating agents which are capable of forming alkyl adducts at 12 different DNA base sites. The relative proportion of alkylation at the nitrogen and oxygen atoms of the purine and pyramidine bases, such as the O^6 position of guanine and the O^4 position of thymine, depends upon the alkylating agent (reviewed by Montesano, 1981).

1.4.3 O^6- alkylguanine

Not all alkylating agents are carcinogens in animal models, but those compounds that are most carcinogenic produce alkyl adducts at the O^6 position of guanine (Loveless, 1969). Work in rodents has also linked exposure to alkylating agents with the presence of O^6-alkylguanine, mutagenicity and carcinogenicity (Goth and Rajewsky, 1974; Newbold et al, 1980; Bartech et al, 1983). Thus, although O^6-alkylguanine is not the most frequent DNA lesion induced by alkylating agents, it is biologically more important in terms of its association with malignant change.

O^6-alkylguanine, unlike native guanine in DNA, is unable to pair with cytosine on DNA replication but mispairs with thymine. This results in a GC to AT transition mutation (Coulondre and Miller, 1977; Eadie et al, 1984), shown schematically in Figure 1.4.3. The mechanism underlying this non-complementary base pairing is still a subject of debate (Loveless, 1969; Swann,1990).

Evidence that these G to A mutations are carcinogenic through activation of oncogenes comes from the several different lines of experimental evidence. Firstly, the finding that single low dose nitrosamines consistently induce mammary tumours in rats which contain G to A mutations in the H-*ras* oncogene (Zarbl et al, 1985), and that nitrosamines produce similar mutations in skin tumours (Brown et al, 1990). Secondly, the finding of G to A mutations in the tumour suppressor gene p53 in rat oesophageal and kidney tumours induced by *N*-nitroso compounds (Ohgaki et al, 1992). The mutations within the H-*ras* proto-oncogene are consistently in the second base of codon 12 with the sequence 5'-CTGGA-3'. In the rat, oesophageal tumours mutations were also restricted to specific bases within two codons in exon 6 of the p53 gene (i.e. the second guanine in CGG [codon 213] and the first guanine in GAG [codon 204]). The specificity of these mutations seems in part to be due to the ability of the nitrosamines (Mitra, 1989; Georgiadis, 1991) to gain access to certain guanine

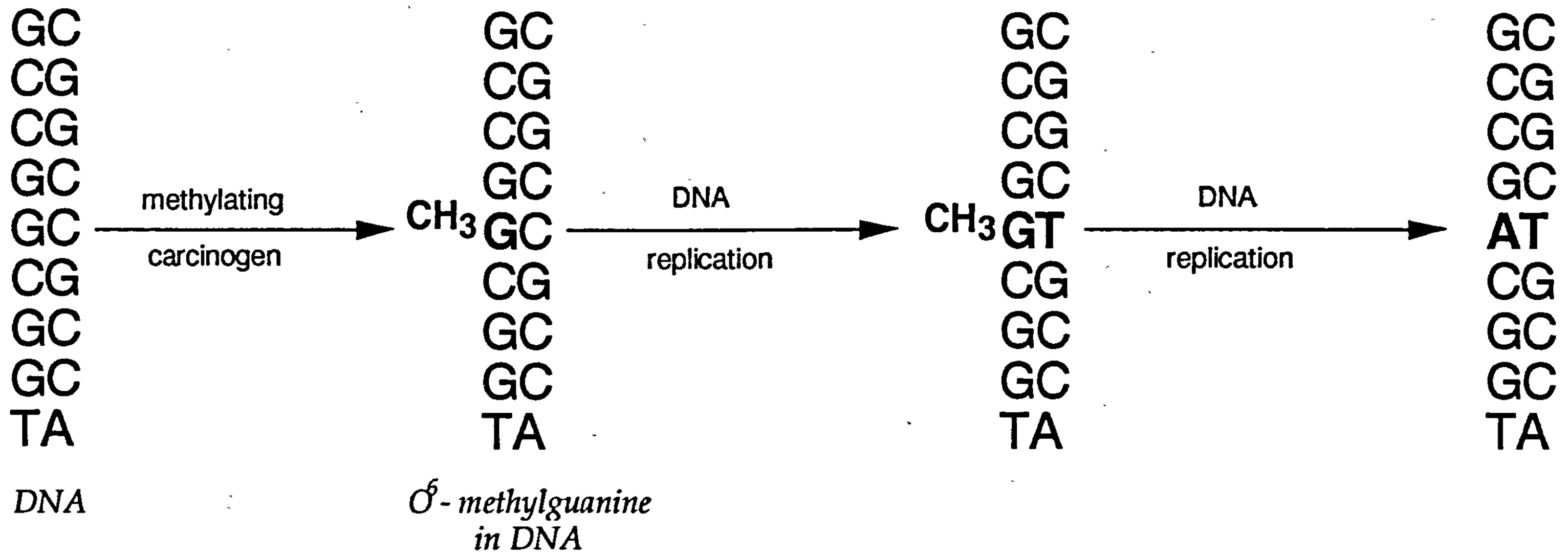


Figure 1.4.3: The mechanism of exposure to alkylating agent leading to a GC toAT transition mutation.

residues and partly the effect of neighbouring bases on miscoding by DNA polymerase (Singer et al, 1989). There is also indirect evidence in *E.coli* for sequence-specificity of DNA repair which may also contribute to the apparent non-random nature of these mutations (Topal et al, 1986; Topal, 1988).

1.4.4 Importance of cell proliferation

Cell proliferation is pivotal to the process of chemical carcinogenesis. DNA replication is needed to convert the promutagenic DNA base lesion O^6-alkylguanine into a mutation. Furthermore, the increased cell turnover increases the number of mutated cells, thus allowing them to overcome growth inhibitory properties of surrounding cells and also increases the chance of further mutations. The importance of cell turnover is reflected in the use of agents or 'promotors' known to cause cell proliferation in most of the models of rodent chemical hepatocarcinogenesis (reviewed by Farber and Cameron, 1980). Agents used include phenobarbitone and carbon tetrachloride which increase the yield of liver tumours produced following a single dose of the chemical carcinogen dimethylnitrosamine (Weisburger et al, 1975). The action of phenobarbitone is probably mediated via direct increased cell turnover and enhanced xenobiotic metabolism whereas carbon tetrachloride causes hepatocyte necrosis which leads to secondary cell proliferation associated with tissue repair. It has been postulated that the cell necrosis with secondary proliferation, or mitogenesis, that is seen following exposure to a carcinogen may in itself be enough both to initiate and promote the appearance of tumours (Ames, 1990). This may explain why, in rodents, the number of HCC forming can be increased by the use of increasing doses of nitrosamines alone; nitrosamines are known to produce hepatocyte necrosis in a dose dependant manner (Solt and Farber, 1976).

Another important agent for increasing cell proliferation commonly used in rodent models of hepatocarcinogenesis is partial hepatectomy. The response of the liver to partial hepatectomy is restorative increased cellular proliferation with DNA synthesis peaking 24 hours later (Mead and Fausto, 1989). Partial hepatectomy, coupled with an exposure to nitrosamines results in the formation of preneoplastic or dysplastic foci in rats, whereas no such foci are observed when the rats receive the same carcinogen with a sham operation (Columbano et al, 1981). In another study, more tumours were observed when nitrosamines were administered at increasing times after the hepatectomy, with most tumours occuring when the carcinogen was given 24-48 hours later, a time when cell proliferation was maximal (Pound and Lawson, 1975).

Figure 1.4.4 shows the mechanism whereby HCC may develop in cirrhotic tissue following exposure to environmental alkylating agents thus potentially explaining why cirrhosis is a risk factor for HCC.

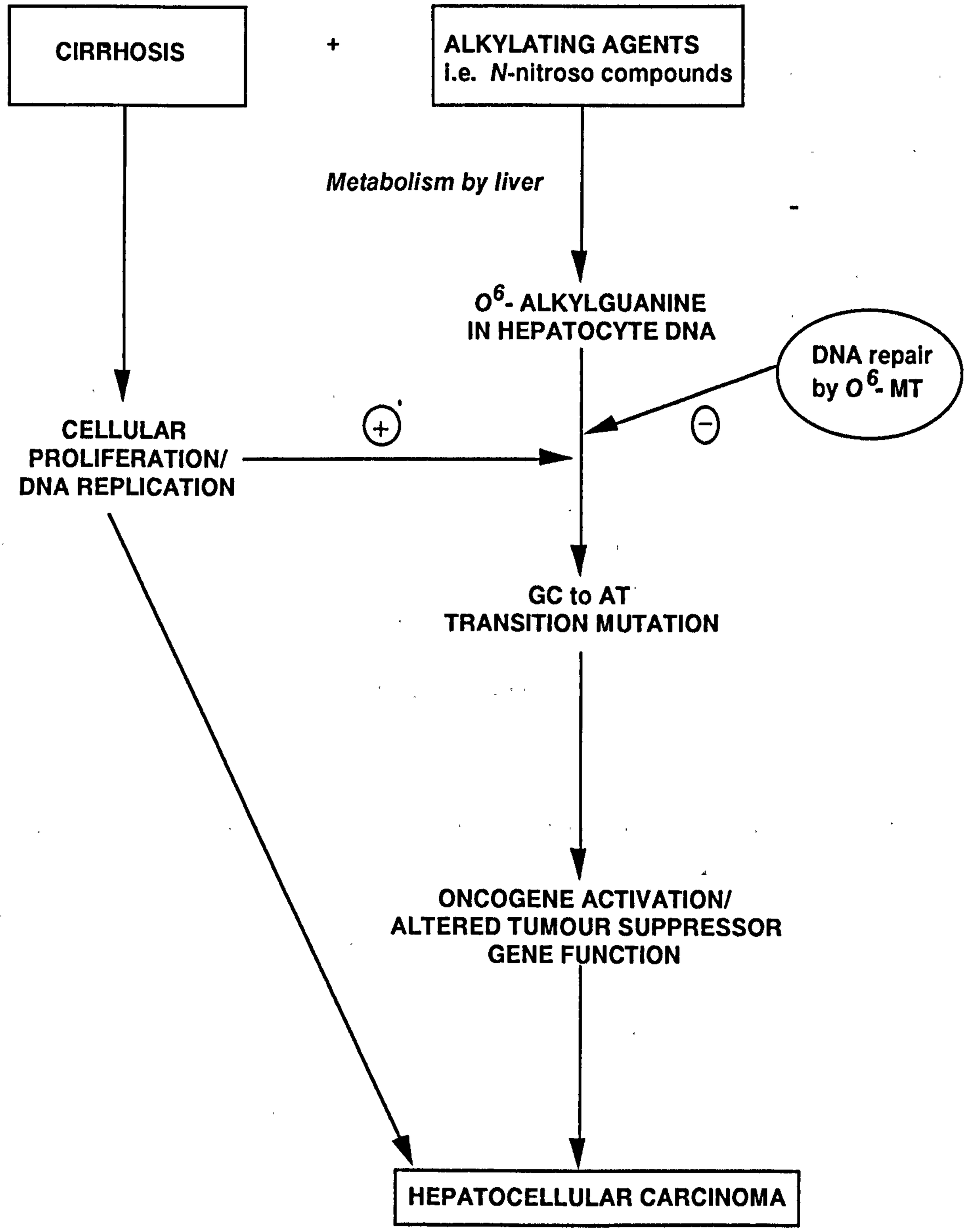


Figure 1.4.4: A possible mechanism whereby N-nitroso compounds, acting on cirrhotic tissue, produce hepatocellular carcinoma.

1.5 DNA repair

DNA repair mechanisms are important in protecting cells from both spontaneous and environmentally induced DNA damage (reviewed by Friedberg, 1985). DNA damage can be caused by chemicals and physical agents such as ultraviolet and ionizing irradiation. Chemicals can damage DNA by the process of alkylation (as discussed above), or the formation of DNA-DNA or DNA-protein crosslinks. There are many repair mechanisms now known to deal with such damage including; (a) those that reverse DNA damage, such as the repair of single strand breaks, (b) excision repair, either nucleotide or base excision, and (c) post-replication repair. Defects in DNA repair mechanisms are linked to the development of some inherited cancers. This is exemplified by xeroderma pigmentosa, where a defect in excision repair of DNA containing thymidine dimers caused by UV light, results in skin cancer. The contribution made by defects in DNA repair to the development of sporadic tumours has still to be determined.

The promutagenic DNA base lesion O^6-alkylguanine, produced by alkylating agents, is repaired by the unusual DNA repair enzyme O^6- methylguanine-DNA methyltransferase (O^6-MT, also abbreviated to MGMT) which acts by removing alkyl groups from the O^6 position of guanine restoring native guanine in DNA prior to DNA replication(reviewed by Pegg ,1990; Strauss,1990).

1.6 O^6- methylguanine-DNA methyltransferase

1.6.1 Background

O^6-MT was first identified in *Escherichia coli* as a M_r 39,000 protein, encoded by the *ada* gene. It consists of two domains separated by a hinge region sensitive to endogenous protease action (Bhattacharyya et al, 1988a). The protein is split by proteolytic cleavage and the resulting M_r19,000 carboxy terminal fragment repairs O^6-alkylguanine. *E.coli* also contains a second gene, *ogt*, that encodes a protein of M_r 19,000. The *ogt* protein displays a 29% homology with the carboxy terminal domain product of the *ada* gene and is also able to repair O^6-methylguanine (I'otter et al, 1987). Methylation of the *ada* protein at cysteine 69 converts the *ada* protein into a strong transcriptional activator of its own gene (reviewed by Lindahl et al, 1988). Such a mechanism is the basis of the so called "adaptive response" whereby levels of the enzyme are induced in response to alkylation damage so increasing the repair capacity of the cell for these adducts. In contrast to the *ada* gene product the *ogt* gene protein is not induced in this manner (Wilkinson et al, 1989).

Similar alkyltransferase enzymes have now also been identified, and the gene encoding the enzymes cloned, in other bacteria (Rebeck et al, 1989), yeast (Sassanfar and Samson, 1990), rodents (Wilkinson et al, 1990) and man.

1.6.2 Function

O^6-MT acts by removing alkyl groups from the O^6 position of guanine in DNA which it transfers to its own specific cysteine residue, so restoring native guanine in DNA, and as a result it becomes inactivated (Foote et al, 1980; Pegg et al, 1983). It is therefore not strictly an enzyme as it only acts once but nor is it an acceptor protein as the protein itself mediates the transfer of the alkyl group from the DNA. The number of O^6-alkyl adducts that can be repaired is therefore limited to the number of O^6-MT molecules available. O^6- methylguanine is the preferred substrate, but longer alkyl groups can also be removed. The repair protein is usually either referred to as O^6-methylguanine-DNA methyltransferase or O^6- alkylguanine-DNA alkyltransferase. The rate of repair decreases with increasing size of the alkyl group and branched chain groups are repaired more slowly than linear alkyl groups (Pegg et al, 1984). Unexpectedly, O^6-benzylguanine, which has a large benzene ring-containing structure, is a good substrate for O^6-MT and therefore rapidly inactivates the protein (Dolan et al, 1990). There is accumulating evidence that longer alkyl adducts are repaired by both O^6-MT and DNA excision repair processes (Bronstein et al, 1992). The exact mechanism by which these longer adducts are repaired is still unknown although it has been suggested that nucleotide excision repair enzymes may be needed for O^6-MT to gain access to DNA through the complex chromatin structure (Bronstein et al, 1992). Double stranded, as opposed to single stranded, DNA is also a preferred substrate for O^6-MT. Repair of O^6-methylguanine is to some extent sequence specific. By using oligonucleotide substrates of different sequence containing alkylguanine, Dolan et al (1988) showed that repair was slowest when O^6-methylguanine is located on the 3'-side of another guanine residue (Dolan et al, 1988). All sequences, however, were repaired such that differences in repair rates are only likely to be important when the number of adducts exceeds the number of O^6-MT molecules (Dolan et al, 1988).

1.6.3 Regulation of levels

The human O^6-MT gene, which is located on chromosome 10 (Rydberg et al, 1990), consists of 5 exons and spans over 170kb, but encodes an mRNA of only 0.95-1.0kb (Nakatsu et al, 1993). Factors affecting regulation of enzyme levels have been analysed using cell lines containing enzyme that is either detectable (so-called "Mer+") or undetectable (so-called "Mer -"). O^6-MT levels are probably transcriptionally regulated as Mer- cells have undetectable levels of mRNA, as determined by Northern blotting, but with no associated gene rearrangements (Pieper et al, 1990; Ostrowski et al, 1991a). Little is known about the regulation of O^6-MT gene transcription. The gene promotor region has been identified but only partially characterised (Harris et al,1991; Harris et al 1992). Gene methylation is, however, one important factor controlling RNA levels. Increasing gene methylation usually correlates with decreasing cellular RNA levels (Cedar, 1988). However, there is a direct correlation between O^6-MT gene methylation and gene expression which may explain how basal O^6-MT levels are re-established following exposure to alkylating agents (Pieper et al, 1991a; Fritz and Kaina, 1992).

O^6-MT comprises only a minor proportion of total cellular protein (approximately 0.00025%) in human liver (Pegg, 1990). This corresponds to about 30,000 to 100,000 molecules per cell in human cell lines (Yarosh et al, 1983; Foote and Mitra, 1984). The normal rate of synthesis and cell turnover of the enzyme in human tissues is unknown. Levels of the *E coli* enzyme increase up to 100-fold in response to alkylation (reviewed by Lindahl et al, 1988). Furthermore, levels of the rodent enzyme have been reported to increase, although only of the order of 3-fold, in response to stimuli such as partial hepatectomy (Pegg et al, 1981a) and non-alkylating hepatotoxins (Pegg and Perry, 1981b). Initial studies on human cell lines suggested that O^6-MT levels could be induced by exposure to alkylating agents, which deplete active enzyme levels, but only of the order of about 3-fold (Waldstein et al, 1982a). However, this has not been confirmed by other groups using the same cell lines (Yarosh et al, 1984; Foote and Mitra, 1984). A recent study using the colorectal cell line HT29 showed no induction of enzyme synthesis following exposure to *N*-nitroso compounds, and that basal enzyme levels are only restored slowly at around 700 molecules/cell/hour (Pieper et al, 1991b). The effect of other exogenous factors such as cell proliferation and ionizing radiation on human O^6-MT levels remains unknown although interestingly, cell cycle changes in O^6-MT levels have been reported (Kim et al, 1986) and the ability of human lymphocytes to restore basal levels of the enzyme following depletion by *N* -nitroso compounds appears to depend on their proliferative state (Gerson, 1988).

1.6.4 Human cell and tissue levels

Human tissues and cells have a much higher content of O^6-MT than equivalent tissues from rodents, of the order of 5-10 fold (Gerson et al, 1986). Enzyme levels in man also differ between organs with the highest levels having been reported in liver and spleen and the lowest in brain (Table 1.6.4).

Table 1.6.4: Normal human tissue O^6-MT levels.

Tissue	Mean O^6-MT levels fmol/mg protein	Reference
Liver (n = 9)	1070	Myrnes et al, 1983
T lymphocytes (n = 10)	359	Gerson et al, 1986.
Colon (n = 10)	140	Myrnes et al, 1983
Stomach (n = 8)	600	Kyrtopoulos et al, 1984
Brain (n = 4)	76	Wiestler et al, 1984

The possibility that deficiency in O^6-MT levels may increase cell and tissue susceptibility to carcinogens has led many groups to measure enzyme levels in human tumour cell lines and tumour

tissue. A striking early observation was the finding of undetectable levels of O^6-MT in a large number of malignant cell lines implicating deficient DNA repair in the process of carcinogenicity (reviewed by Strauss, 1990). However, the discovery of an O^6-MT deficient tumour cell line which had been derived from a cell containing detectable levels of enzyme has led to the suggestion that loss of O^6-MT may occur during culture; ie, either cell culture selects for a small population of cells that lack O^6-MT activity or transformation leads to loss of activity. The latter is supported by the observation that virally transformed cells are more likely to lack O^6-MT than other human tumour cell lines (Day et al, 1980). It is also of interest that cells in culture can spontaneously either stop expressing O^6-MT (Green et al, 1990; Day et al, 1991) or spontaneously start producing the enzyme (Arita et al, 1990).

The existence of a large number of Mer$^-$ tumour cell lines has led to O^6-MT levels being measured in a range of human tumours (Citron et al, 1991; Chen et al, 1992; Myrnes et al, 1984). These studies have indicated that unlike tumour cell lines there are only a few tumours, such as brain astrocytomas, colon carcinoma and stomach carcinoma in which O^6-MT cannot be detected (Citron et al, 1991). There is also a wide interindividual variation in enzyme levels from the same tumour type (Citron et al, 1991; Chen et al, 1992). Studies comparing O^6-MT from tumours with levels in surrounding tissue have provided conflicting results. Citron et al (1992) showed no difference in enzyme levels but Myrnes et al (1984) suggested that less enzyme was present in the tumour compared to the surrounding tissue.

1.6.5 Depletion of O^6- MT and carcinogenesis

There is increasing evidence that the cellular level of O^6-MT is an important factor in determining the sensitivity of cells to mutations and malignant transformation. The frequency of mutations following exposure to alkylating agents is higher in cells with lower levels of the DNA repair enzyme (Maher et al, 1990). It has also been shown that tumour cell lines which do not express O^6-MT are more sensitive to alkylating agent induced cytotoxicity (Scudiero et al, 1984) but that introduction of the *E coli ada* gene into these cells results in increased resistance to such compounds (Ishizaki et al, 1986). Furthermore, the presence of O^6-MT appears to protects cells from spontaneous G to A mutations, thought to be due to endogenous O^6-alkylguanine possibly produced following nitrosamine exposure (Aquilina et al, 1992). Finally, Dumenco et al have recently published an important study showing that transgenic mice expressing the human O^6-MT gene in their thymus were protected from forming thymic lymphomas containing G to A mutations in the H *ras* oncogene following exposure to nitrosamines (Dumenco et al, 1993).

1.7 Oncogenes, tumour suppressor genes and growth factors

Cellular oncogenes are components of signal transduction pathways their products include; extracellular growth factors and hormones, growth factor receptors on the cell membrane, cytoplasmic binding proteins and nuclear transcription factors. These pathways are pivotally involved in cellular proliferation and differentiation. Abnormalities of expression of these gene

products are important components in the multistep process of carcinogenesis (reviewed Bishop, 1991).

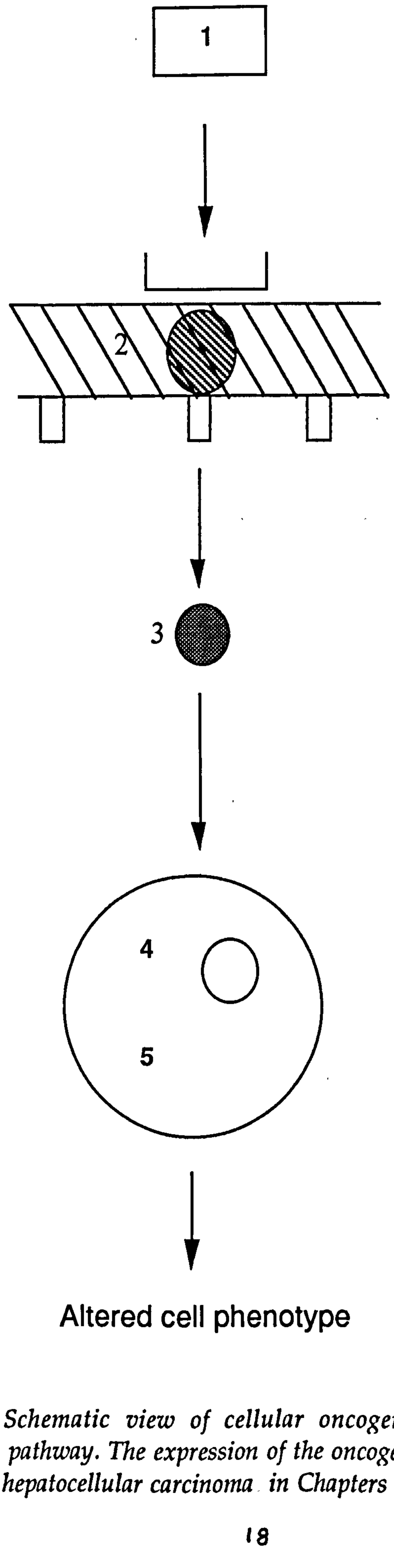
There are now over 60 recognised oncogenes (Bishop, 1991) but surprisingly few have been studied and implicated in human hepatocarcinogenesis. Oncogenes may be activated by mutations or gene amplification. Mutations include point mutations, such as the G to A mutation produced by O^6-alkylguanine, and deletions and rearrangements. The *ras* oncogenes (N-*ras*, K-*ras* and H-*ras)* which encode cytoplasmic GTP binding proteins, are an example of oncogenes that are activated by point mutations, and are the oncogenes most frequently implicated in the development of human tumours (Bos, 1989). Several studies have shown enhanced expression of *ras* genes in rat liver tumours (Makino et al, 1984; Chander et al, 1987) and elevated p21 *ras* protein has been detected in human cirrhotic nodules and HCC (Nonomura et al, 1987). However only 4 out of 20 (20%) human HCC have been shown to have mutations in codons 12 and 61 of the ras oncogenes and none of these were G to A (Challen et al, 1992a).

Recognised tumour suppressor genes include the retinoblastoma gene (RB), p53 and DCC (deleted in colorectal cancer) gene. Alteration of tumour supressor gene function occurs through point mutations and allele loss with resulting loss of tumour suppression. The normal p53 gene product is believed to be involved in controlling entry of cells into S phase of the cell cycle (Harris, 1991). p53 mutations are common in human cancers and a significant number are G to A mutations (reviewed by Hollsteine et al, 1991).

The number of recognised peptide growth factors are also increasing (reviewed Cross and Dexter, 1991). An important group of growth factor receptors are those encoded by the c-*erb* B1-3 proto-oncogenes (reviewed Prigent and Lemoine, 1992). The protein products of these proto-oncogenes are a family of transmembrane proteins, which are often overexpressed in human tumours. c-*erb* B-1 encodes for the epidermal growth factor (EGF) receptor (EGFR) which is also the receptor for transforming growth factor alpha (TGF–α) (Todaro et al, 1980). C-*erb* B-2 encodes a receptor, for which the ligand is still unknown, whose overexpression correlates with prognosis in breast carcinoma (reviewed by Maguire and Greene, 1989). The third member of this family of growth factor receptors is the c-*erb* B-3 (Kraus et al, 1989; Plowman et al; 1990) protein product which is also overexpressed in human tumours, such as breast and pancreas (Lemoine et al, 1992; Lemoine et al, 1993). Figure 1.7 illustrates the cellular location of TGF-α and the protein products of c-*e rb* B-2, ras, and p53 which can all be considered as components of the cells' signal transduction pathway affecting growth and differentiation.

1.8 Aims of the study

The overall aim of this study was to look at the molecular mechanisms involved in human hepatocarcinogenesis in Britain, in particular to assess possible mechanisms involved in the malignant transformation of cirrhotic tissue and to assess factors potentially involved in tumour growth.



1. Growth factors

EGF/TGF-α
PDGF
FGF
IGF I/II

2. Growth factor receptors

c-*erb* B-1
c-*erb* B-2
c-*erb* B-3

3. GTP binding proteins

ras

4. Nuclear

c-*myc*
fos
myb

5. Tumour suppressor gene

Rb
p53

Altered cell phenotype

Figure 1.7: Schematic view of cellular oncogenes as components of the signal transduction pathway. The expression of the oncogene products outlined in bold will be examined in hepatocellular carcinoma in Chapters 6-8 of this study.

In view of the known geographical differences in the aetiology and possible mechanisms of hepatocarcinogenesis the first aim of the study was to establish the clinical features of hepatocellular carcinoma in Northern England. Particular attention was paid to the prevalence of risk factors previously reported from other geographical areas, namely the presence of hepatitis B infection and cirrhosis.

The second aim was to measure the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (O^6-MT) in cirrhotic tissue and to compare levels with those in non-cirrhotic liver. The central hypothesis of this investigation was that deficient repair of O^6-methylguanine in cirrhotic tissue might increase the likelihood of G to A mutations in oncogenes and tumour suppressor genes involved in the development of hepatocellular carcinoma. In order to measure O^6-MT in small liver biopsy samples a novel enzyme microassay was developed. The relationship between enzyme levels in lymphocytes and liver from individual patients was also assessed to determine whether any deficiency in O^6- MT levels was both tissue - and disease-specific.

The third aim was to develop polyclonal antibodies to three different regions of the DNA repair protein O^6-MT to use such reagents to establish the cellular distribution of O^6-MT in normal and diseased liver by immunohistochemistry. In addition these antibodies could be used in immunoblots of extracts separated by SDS-PAGE to characterise different molecular weight forms of the enzyme in cirrhotic and non-cirrhotic liver and in lymphocytes. SDS-PAGE of radiolabelled liver and lymphocyte extracts followed by fluorography could therefore be used to confirm the enzyme assay findings.

The fourth aim was to look at expression of the tumour suppressor gene p53 in British HCC which is known to be altered by mutations which include G to A transition mutations produced by the inability of cells to repair O^6-methylguanine. The final aim was to look at growth factors that might also be involved in hepatocarcinogenesis, namely the expression of transforming growth factor alpha and the growth factor receptor c-*erb* B-2 using immunohistochemistry.

The tissue used in this study came from two sources: prospectively collected liver biopsy tissue was used to examine the DNA repair enzyme in cirrhotic and non-cirrhotic liver, whereas formalin-fixed paraffin-embedded archival liver biopsy tissue was used to study the expression of p53, c-*erb* B-2 and TGF-α in hepatocellular carcinoma.

Chapter 2

Clinical Features Of Hepatocellular Carcinoma

2.1 Introduction

Previous studies of the clinical features of HCC have shown marked geographical differences (reviewed by Johnson,1987). HCC differs between high and low incidence areas in the age of tumour onset, aetiological factors, extent of tumour at presentation and survival (Liver Cancer Study Group Of Japan, 1987; Calvert et al, 1990). This suggests that the mechanisms of hepatocarcinogenesis are non-uniform. Although large studies of the features of HCC have been reported from areas of high prevalence, studies from the United Kingdom (UK) have been either small or have been carried out by tertiary referral centres in areas where there are large population movements. Table 2.1 shows that in the UK these studies have been principally undertaken in two London tertiary referral units. Although there is also a large study from Glasgow, this was based on post mortem data and so the findings can not be directly compared to antemortem data from other areas.

Table 2.1: Studies detailing the clinical features of HCC in the United Kingdom

Area	Number	Type of study	Reference
Glasgow	100	Post mortem	MacSween , 1974
London (Kings)	96	Retrospective	Johnson et al, 1978
	150	Cirrhosis/non-cirrhosis	Melia et al, 1984
	42	Prospective	Zaman et al, 1985
	49	Immigrants/non-immigrants	Zaman et al, 1986
London (Royal Free)	41	Retrospective	Dunk et al, 1988

The aim of this study was to establish the clinical features of HCC in a relatively stable population from the North East of England, thus identifying possible aetiological factors in the population in which the molecular mechanisms of hepatocarcinogenesis were to be studied further.

2.2 Patients and Methods

The study population comprised 110 consecutive patients with a histological diagnosis of primary hepatocellular carcinoma (HCC) seen at Freeman Hospital, Newcastle upon Tyne from 1978 to 1992. Full clinical details including the results of investigations, treatment and survival were obtained retrospectively from case notes. Clinical records were also obtained from the referring hospital in cases of regional referrals. 105 patients were Caucasian of whom 99 had spent most of their life in the North East of England.

Statistical analysis

All data collected were documented on a standard proforma, designed for transfer of information to the Northumbria Universities Multiple Access Computer (NUMAC). Computer instructions were written to read this raw data and create a systems file using SPSS-X (SPSS-X Batch System, SPSS Inc.Chicago, Ilinois, USA). Validation procedures were carried out to ensure accurate data transcription. Non-parametric statistical methods were used for comparisons between groups. Survival curves were calculated by the Lee-Desu statistic (Lee and Desu, 1972). The effects of age group, treatment and tumour stage were calculated by the Cox's regression model (Cox, 1972).

2.3 Results

2.3.1 Age and sex

The median age of the 110 patients was 64 years with a range of 16 to 83 years (Figure 2.3.1). The male to female ratio was 7 to 3.

2.3.2 Symptoms and signs at presentation

The median time from the onset of symptoms to presentation was 8 weeks (range 1 to 104). 7 patients were asymptomatic at presentation having been identified through α-fetoprotein screening. Symptoms at presentation were non-specific with abdominal pain occuring in 69% but only localised to the right upper quadrant in 33% of these cases. Hepatomegaly was a common finding at presentation but evidence of chronic liver disease (CLD) such as cutaneous stigmata and /or splenomegaly was found in only a third of patients. (Table 2.3.2). Hepatic decompensation in the form of ascites was not uncommon (34%) but encephalopathy and variceal bleeding were rare (n=4 and n=2 respectively)

2.3.3 Aetiology

Only 25% (n= 27) of patients were known to be cirrhotic at presentation although following subsequent investigation cirrhosis was diagnosed in 76% (75/99) of patients (65 cases based on histology of non-tumour affected liver and10 on clinical criteria where histology of tumour alone was available). The presence or absence of underlying cirrhosis could only be determined in 99 patients as in the other 11 cases only tumour was present on liver biopsy and clinical criteria were unhelpful.

The commonest types of cirrhosis were those associated with alcohol abuse (diagnosed on the basis of a history of prolonged alcohol ingestion of more than 80g/day and/or histological evidence of alcohol-induced injury), hepatitis B infection (the presence of HBsAg or HBcAb alone in the absence of alcohol abuse) and ' cryptogenic' (Figure 2.3.3). A diagnosis of cryptogenic cirrhosis was made, in the absence of hepatitis C screening, if there was no obvious aetiological factor. Hepatitis C virus antibody testing only became available during the last 18 months of the study. Sera from 17 patients were tested for hepatitis C and 4 (23%) had antibodies using the second generation RIBA test (Abbott). Cirrhosis in these patients had previously been diagnosed as alcoholic (n=1) and

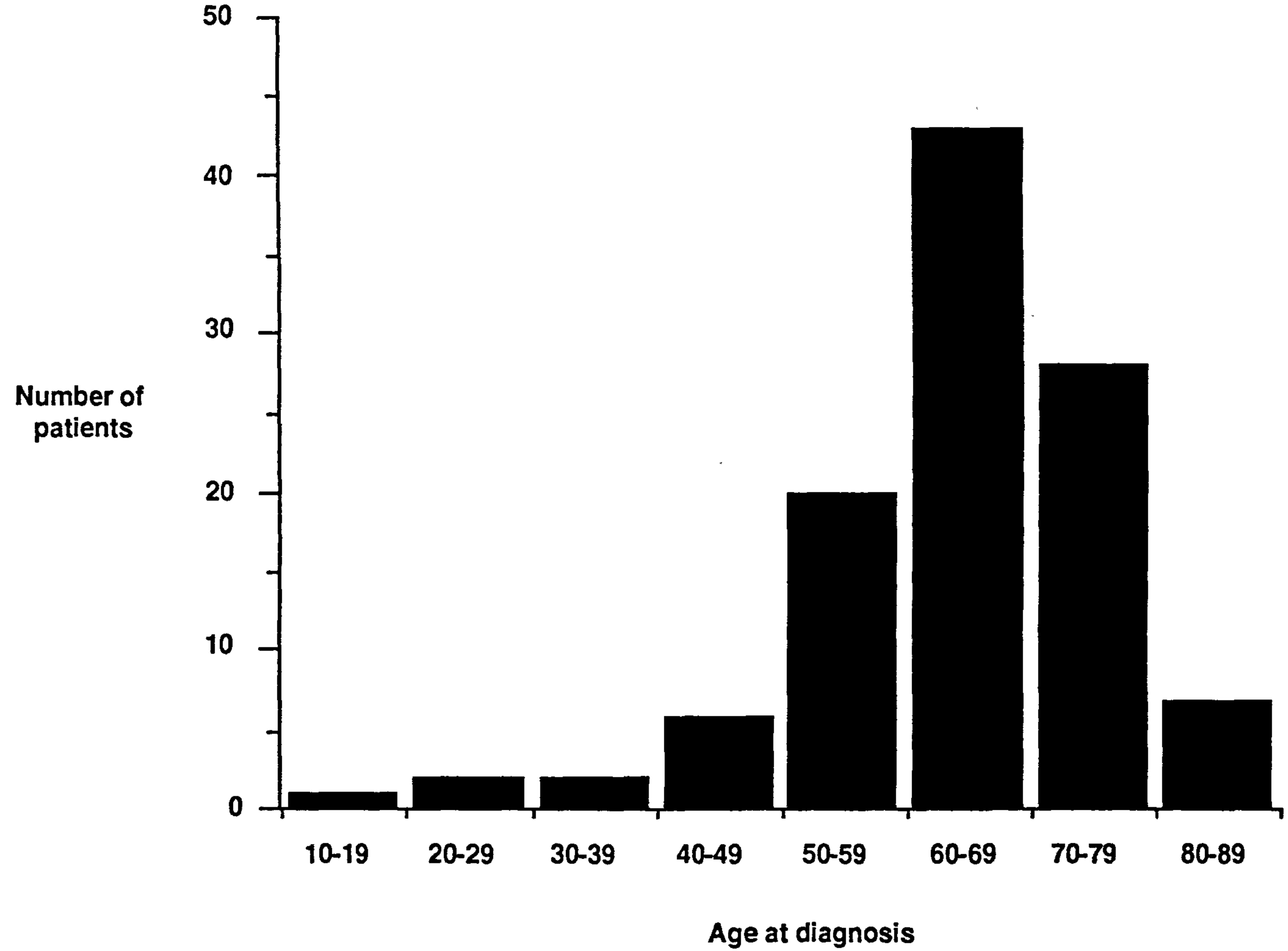


Figure 2.3.1: Age distribution of 110 patients with HCC.

Table 2.3.2: Clinical features at presentation.

Symptoms and signs	**Number (%) of patients**
Abdominal pain	76 (69%)
Weakness	74 (67%)
Anorexia	64 (58%)
Weight loss	59 (54%)
Nausea	45 (41%)
Hepatomegaly	91 (83%)
Jaundice	41 (37%)
Cutaneous stigmata of CLD	38 (35%)
Ascites	37 (34%)
Splenomegaly	23 (21%)

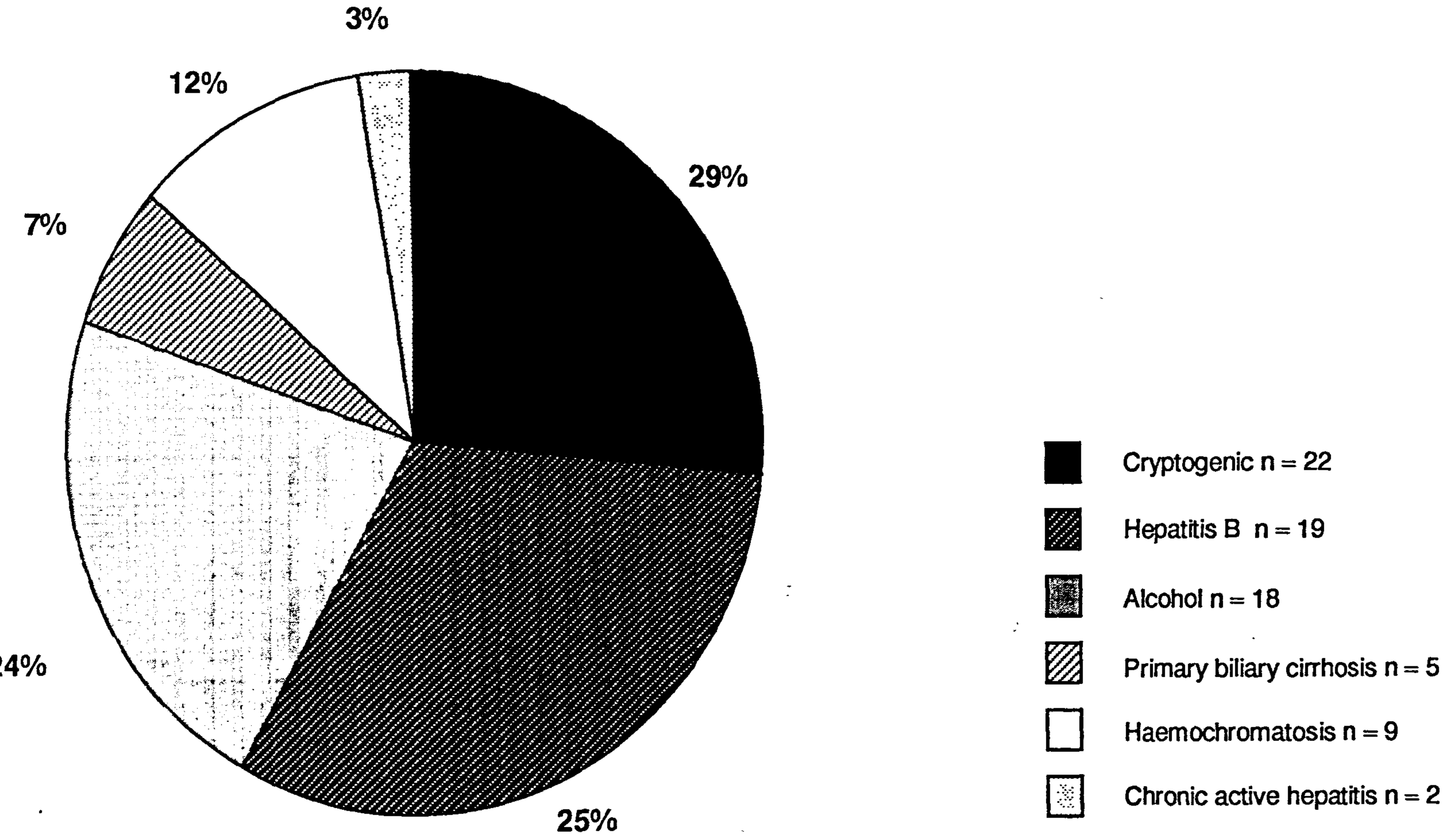


Figure 2.3.3: Aetiology of underlying cirrhosis

cryptogenic (n=3). Serological markers of hepatitis B infection (HBsAg or HBcAb alone) were present in 29% (n=32) of patients; HBsAg in 9% (n=10) and HBcAb alone in 22% (n=22). Hepatitis B-related cirrhosis was present in 19 cases. The other 13 cases included one patient with HBsAg and no cirrhosis, and 12 cases with HBcAb alone. Insufficient normal liver was available to establish the presence or absence of cirrhosis in 6 of these cases and the other 5 cases were cirrhotic but the aetiology was considered to be haemochromatosis (n=1), alcohol (n=2), and autoimmune chronic hepatitis (n = 2) based on a combination of serum biochemistry and immunology, and histological findings.

Cirrhotic patients were more likely to be male than non-cirrhotic patients and had a worse prognosis (Table 2.3.3). No fibrolamellar tumours, which occur characteristically in predominantly younger non-cirrhotic females and have a better prognosis, were seen in this study.

2.3.4 Investigations

The median haemoglobin concentration was 12.7g/dl and only 2 patients had an haematocrit of greater than 0.5 . The prothrombin time was prolonged in 54% of patients but was prolonged to greater than 4 seconds above normal in only 9%. Most patients had abnormal liver function tests but no pattern (hepatitic or cholestatic) predominated (Table 2.3.4). Hypoglycaemia at presentation was only diagnosed in 2 patients. Both patients had symptomatic hypoglycaemia with a low random serum glucose level. Fasting glucose levels were not performed on any of the patients in the study.

27 patients were known to be cirrhotic at diagnosis and thus potential candidates for α-feto protein (α-FP) screening at six monthly intervals to detect early treatable tumours. Of these patients, 22 had a raised α-FP and could therefore have been diagnosed through screening. However eleven of this group had not received screening and 4 patients had only had the occasional α-FP level measured; the diagnosis of HCC in these patients was made because of symptoms. Thus only 7 patients were asymptomatic and diagnosed as having HCC through screening. At diagnosis 13% (n=14) patients had an α-FP of less than 10ng/ml. In the remaining patients the α-FP levels were between 10-200ng/ml in 24%, 200-500ng/ml in 16% or greater than 500ng/ml in 47%.

Ultrasound scanning was performed in 83% (n=91) of cases, CT scans in 55% (n=60) and angiography in 48%(n=53). The ultrasound was abnormal in 93% (n=85) of cases showing either a single hepatic mass (n=43), diffuse hepatic infiltration (n=15) or multiple tumour nodules (n=27). All CT scans were abnormal with a single defined tumour mass reported in 22 cases and multiple tumour nodules reported in 38 cases.

2.3.5.Staging

Patients were staged according to Okuda (Okuda et al, 1986) at diagnosis on the basis of four adverse prognostic features (bilirubin > 50mmol/l, albumin < 30g/l, presence of ascites and tumour size >50% of liver bulk on an anterior-posterior view of a colloid isotope scan). Patients in group 1 had

Table 2.3.3: Comparison of clinical features in cirrhotic and non-cirrhotic patients.

Clinical features	Cirrhotic (n=75)	Non-cirrhotic (n =24)
Median age (yrs)	64	62
Male sex	76%	48%
HBsAg positive	12%	4%
αFP > 500IU/1	40%	60%
Median survival (weeks)	9	16

Table 2.3.4: Presenting biochemical and haematological investigations.

Test (units)	Mean	Range	% abnormal	Normal range
Haemoglobin (g/dl)	12.7	6.6-18.2	31	14-17 (male) 12-15.5 (female)
White cell count ($x10^9$/l)	9.2	2.7-44	29	4-11
Platelets ($x10^9$/dl)	242	21-677	24	150-350
Prothrombin time (sec)	16	11-65	82	15
Bilirubin (µmol/l)	51	3-550	60	<17
Aspartate transaminase (IU/l)	119	15-549	79	<40
Alkaline phosphatase (IU/l)	354	30-1608	82	<130
Albumin (g/l)	34	22-45	14	30-50

none of these features, those in group 2 had one or two of these features and those in group 3, three or four. In this study 23% were stage 1, 63% stage 2 and 13% stage 3.

The size of the tumour was demonstrated by radiology in 63 cases and was greater than 5cm in 79%. The tumour was considered multifocal in 46% (n=51). In the 59 cases considered to have unifocal disease the tumour was limited to the right lobe in 51 and the left lobe in only 8 cases. It is likely that these results underestimate the extent of liver involvement by HCC as the findings are obtained from a retrospective review of radiology reports of procedures performed by different radiologists.

2.3.6 Treatment

Fourteen patients were considered to have surgically resectable disease and underwent laparotomy. However, at operation curative resection was not possible in any of these. Nine patients subsequently had adjuvent chemotherapy. 27 (25%) patients were treated conservatively. The other 69 patients received chemotherapy with intrahepatic adriamycin /lipiodol (n= 36) or intravenous chemotherapy, which included adriamycin, CB3717 or VP16 with 5 -flurouracil.

2.3.7 Prognosis

The overall median survival was 13.1 weeks. Dependant factors affecting survival included age, Okuda stage and treatment. The median survival of patients aged over 65 years was 10.5 weeks compared to 18.5 weeks for those under 65 years (p=0.02). The median survival of Okuda stage 1 patients was 16.5 weeks, while that for Okuda stage 2 was 13 weeks and for stage 3 patients was 7.6 weeks (p=0.03). Due to the small numbers treated with surgery alone and surgery combined with chemotherapy these groups were combined with chemotherapy when analysing survival. The median survival of those treated conservatively was 6.4 weeks compared to a median survival of 15.1 weeks in the treatment group (p=0.008). Since the older age group were more likely to be treated conservatively the interaction of age group (<65 or >65 years) and treatment (conservative v any treatment) was examined by a Cox regression model (Cox, 1972). Using this model the adverse effect on survival of age group disappears when adjusted for treatment (Table 2.3.7).

2.4 Discussion

This study confirms the association of HCC with older age, male sex, hepatitis B viral (HBV) infection, and cirrhosis which are believed to be important aetiological factors in hepatocarcinogenesis. Increasing age and male sex are universal risk factors world-wide although the median age of onset of HCC is 10 years lower in high incidence areas such as South Africa (Kew and Geddes, 1982).

Serological markers of hepatitis B infection however were only present in 29% of this British patient population which contrasts with the high prevalence of HBV in HCC patients from high incidence areas (reviewed Chapter 1). These values may underestimate the role of the virus in HCC as HBV DNA has also been detected in tumour liver and surrounding cirrhotic liver of patients with HCC in

Table 2.3.7: Effect of age group , Okuda stage and treatment on survival. Cox regression model. Dependant variable is survival; independant variables are age group (<65, 65 or greater), Okuda stage (I,II,II) and treatment (conservative, any treatment).

Variable	Coefficient (ß)	Standard error (SE)	ß/SE	Reliable risk (RR)	Confidence interval 95% for RR
Model 1					
Age group	0.197	0.208	2.383	1.643	1.09-2.41
Model 2					
Okuda	0.520	0.221	2.357	1.68	1.09-2.59
Age group	0.432	0.210	2.01	2.01	1.01-2.30
Model 3					
Treatment	1.203	0.258	4.654	3.33	2.01-5.53
Age group	0.236	0.223	1.061	1.26	0.82-1.96

the absence of serological HBV markers (Paterlini et al, 1993). Other environmental agents that have been implicated in the pathogenesis of HCC include alcohol and hepatitis C. Alcohol is only associated with an approximately 4 fold increased risk of developing HCC (Vecchia et al, 1988 Poynard et al,1991). It is, therefore, more likely that it acts as a cofactor with other hepatocarcinogens (reviewed by Bassendine, 1986). Hepatitis C infection is related to HCC but the prevalence of infection in Britain is low and is unlikely to prove a major contributory factor; this is in contrast to countries in Southern Europe (Sirchia et al, 1989). Thus other, as yet unrecognised, environmental toxins need to be considered as potential hepatocarcinogens in Northern Europe. The finding of cirrhosis in 76% of patients with HCC is in agreement with the high incidence of cirrhosis associated with these tumours world-wide (reviewed Chapter 1).

In this study symptoms at presentation were generally nonspecific which is similar to findings reported from North America (Chlebowski et al, 1984) and the Far East (Liver Cancer Study Group Of Japan, 1987). The presence of abdominal pain, however, present in 69%, should arouse suspicion of a developing HCC in an at risk patient. Interestingly paraneoplastic phenomena, such as hypoglycaemia and polycythaemia, were rare. This contrasts with reports of hypoglycaemia in up to 30% of patients with HCC in high incidence areas (McFadzean and Tse Tse, 1956). The overall picture of HCC in Britain is of a tumour that presents late and often on the background of undiagnosed cirrhosis. This is exemplified by the finding of multifocal tumour in 46% of patients in this study with 80% of tumours over 5 cm in diameter. Moreover, this study probably underestimates the extent of disease as many of these assessments was made only on the basis of ultrasound and isotope liver scans. The recent availability of lipiodal angiography, lipiodal being retained by the tumour and detectable on a CT scan performed two weeks after angiography, has led to an increase in the detection rate for multifocal tumours.

The overall survival of patients in this series was only 3 months. This is comparable with survival rates reported from Europe (Calvert et al, 1990) and for untreated patients from Japan (Nagasue et al, 1984). However, overall the prognosis of HCC is much better in Japan and this probably reflects the fact that 50% of tumours are operable at diagnosis (Liver Cancer Study Group Of Japan, 1987). In contrast, all tumours in this study were unresectable, based preoperatively on the presence of multifocal tumour or tumour greater than 5cm, or on operative findings. The reason for the late presentation and poor prognosis of HCC in Western countries is still unclear. One factor may relate to the presence of cirrhosis which is an adverse prognostic indicator in low incidence areas (Melia et al, 1984; Smalley et al, 1988) in contrast to high incidence areas where the presence or absence of cirrhosis has no effect on the course of the disease (Kew and Geddes, 1982; Nagasue et al, 1984). Another factor contributing to the early detection of HCC in Japan has been the introduction of screening for high risk cirrhotics patients (Tanaka et al, 1987). Screening has been shown to improve the percentage of resectable tumours and hence the prognosis in HCC (Liver Cancer Study Group of Japan, 1990). However a recent study from Italy failed to show any increase in the rate of detection of potentially curable tumours using α-FP and realtime ultrasound screening in known cirrhotics in a low incidence area (Colombo et al, 1991). An important limitation to screening in Britain is the

finding that only 25% of patients were known to be cirrhotic at the time of diagnosis, which is much lower than that reported from Southern Europe and Japan (77% and 54% respectively). Consequently a significant proportion of British patients with HCC would not have have been candidates for screening.

HCC in Britain differs from that in high prevalence areas, such as China, in that it is less likely to be associated with hepatitis B infection or aflatoxin exposure, and more likely to present at an advanced stage. In contrast cirrhosis appears to be an important risk factor for HCC worldwide. This clinical picture suggests the existence of different (but overlapping) mechanisms of hepatocarcinogenesis between Britain and areas of high tumour prevalence. Studies outlined in subsequent chapters of this thesis concentrate firstly, on why cirrhosis may be a risk factor for HCC and secondly, on factors potentially contributing to the aggressive growth of these tumours in northern Britain. Aflatoxin and hepatitis B appear unikely to play a major role in hepatocarcinogenesis in Britain. It is possible that DNA damage in cirrhotic tissue may be induced by other environmental carcinogens, in particular the ubiquitous alkylating agents. The hypothesis that deficient repair of such DNA injury, by the DNA repair enzyme O^6-methylguanine -DNA methyltransferase, occurs in cirrhosis predisposing to the development of HCC is explored further.

2.5 Summary

Clinical Features Of HCC In North-East England

Age (median)	65 yrs
Male : female	7 : 3
Cirrhotic - known at presentation	25%
Cirrhotic - after investigation	76%
Hepatitis B markers	29%
Stage at presentation	>5cm and multifocal
Survival (median)	13.1 weeks

Chapter 3

DNA Repair By O^6 - methylguanine - DNA methyltransferase In Cirrhosis

3.1 Introduction

Despite the well known association between cirrhosis and HCC the role of cirrhosis in the development of HCC remains unknown. The possibility that deficiency in DNA repair mechanisms in cirrhotic tissue may make it more susceptible to damage by environmental carcinogens has not previously been addressed.

O^6 - methylguanine - DNA methyltransferase (O^6-MT) repairs the promutagenic DNA lesion O^6-methylguanine, produced by environmental alkylating agents, by removing the methyl group and transferring it to its own specific cysteine residue (reviewed Pegg et al, 1990). As a result it becomes inactivated and is not regenerated, thus in the absence of new enzyme synthesis the total cellular level of O^6-MT represents the DNA repair capacity of the cell for this promutagenic lesion (Foote et al, 1980; Pegg et al, 1983). O^6-MT appears to require no cofactors and has an optimum pH of about 7.8-8.5 (Pegg et al, 1983; Bouldon et al, 1987). The rate of repair is reduced by unmethylated DNA probably because of binding of O^6-MT to this DNA (Bouldon et al, 1987). It is also strongly inhibited by metal ions such as Cu^{2+}, Hg^{2+}, Zn^{2+} (Scicchitano et al, 1987, Bhattacharyya et al, 1988b). Such inactivation can be inhibited by dithiothreitol which is consistent with the reaction of these metals with the thiol group of the methyl acceptor cysteine. Dithiothreitol is therefore added to buffers in assays of O^6-MT to reduce enzyme loss. Several methods have been developed to measure the level of O^6-MT in tissue extracts (reviewed by D'Incalci et al, 1988). These can be divided into two groups; (a) indirect assays, which measure the disappearance of O^6-methylguanine from methylated DNA after reaction with the O^6-MT containing cell extract of interest, and (b) direct assays, which measure the transfer of radiolabelled methyl groups from a prepared O^6-methylguanine substrate to the protein fraction of an O^6-MT-containing cell extract. In the indirect assays, O^6-methylguanine removal is monitored by HPLC or immunoassay of the acid hydrolysed DNA reaction product. In the direct assays, radiolabelled methylated protein is separated from the repaired DNA by either proteinase solubilisation of protein, or removal of DNA by acid hydrolysis and washing with the radiolabelled protein being retained on glass filters.

The level of O^6-MT in normal human liver tissue has previously been measured in several small studies (Table 3.1). These have shown that liver contains higher amounts of the enzyme than other human tissues but have also demonstrated that there is substantial inter-individual variation as has been described for other tissues (Table 3.1). Measurement of O^6-MT in any tissue is made difficult by the requirement for large tissue samples because of the low enzyme abundance and relative instability. O^6-MT has therefore been hitherto quantified in the liver using tissue obtained at operation rather than from percutaneous liver biopsies; this is reflected in the small numbers of patients studied to date. There are no previous studies looking at the DNA repair enzyme in cirrhotic liver or non-cirrhotic diseased liver.

The initial aim of this study was to establish reliable methodology for measuring the enzyme in small human liver biopsy specimens (5-15mg). O^6-MT was then measured in biopsy samples from

Table 3.1: Mean levels of O^6-MT reported in normal human liver.

n	O^6-MT (fmol/mg protein)	Range (n-fold)	Reference
10	873	4.5	Pegg et al, 1982
9	1070	7.8	Mymes et al, 1983
5	394	2 - 3	Hall et al, 1985
8	480 (55*)	2 - 3	Gerson et al, 1985

* O^6-MT expressed as fmol/μg DNA

normal, and diseased (non-cirrhotic and cirrhotic) liver in order to establish whether DNA repair by O^6-MT was impaired in cirrhotic liver. It had previously been reported that lymphocyte O^6-MT levels correlated with enzyme levels in gastric tissue (Kyrtopoulos et al, 1990). Lymphocyte levels were, therefore, also assayed and compared to liver levels to assess whether they could act at a surrogate marker for levels in normal and diseased liver.

3.2 Clinical material and methods

3.2.1 Development of assay for liver biopsies

Reagents

Buffer A	50mM Tris (Gibco/BRL)/HCl (BDH)(pH 8.3), 0.5mM EDTA (BDH), 1mM dithiothreitol (Calbiochem)
CTAB	3% (w/v) cetyltrimethylammonium bromide (BDH, laboratory reagent grade)
EDTA/DNA	80mM EDTA (BDH) /HCl (pH6.0), 200µg calf thymus DNA (Sigma)
Proteinase K	ImM $CaCl_2$ (BDH), 50µg proteinase K (Sigma type XI)

Unless indicated all reagents were of AnalaR grade or highest purity obtainable.

Tissue

Three different sources of normal human liver were used: (1) caderveric renal transplant donors (samples stored at -80°C for less than 1 year), (2) patients undergoing cholecystectomy (samples stored at -80°C for 5 years), (3) post mortem liver obtained within 4 hours of death. Tissue from patients undergoing cholecystectomy which had been repeatedly thawed and refrozen was also available for study.

Tissue extraction

Extracts of liver were prepared by homogenising 5-15mg of tissue in 1ml of Buffer A using a micro-homogenizer (Omni, USA). Working on ice as far as possible, homogenisation was achieved using 2 x 5 second sessions separated by a 30 second interval. The amount of tissue used was estimated by comparing it's size with previously weighed biopsy samples, as preliminary work had shown that direct weighing of the tissue resulted in loss of enzyme activity, presumably caused by tissue thawing. 10µl of homogenate was removed for assay and the remaining homogenate was centrifuged at 15000xg for 20 min at 4°C. The supernatant (designated 'tissue extract') was removed and used immediately in the assay. The remaining pellet was resuspended by homogenisation in 400µl of buffer A and assayed to ascertain enzyme extraction efficiency. During the procedure all tissues and extracts were kept at 4°C on ice. The enzyme assay was always performed immediately following extract preparation.

Enzyme assay

O^6-MT levels were determined using a sensitive direct enzyme assay (Major et al, 1991). Tissue

extracts were diluted to a final volume of 200µl and then incubated with 10µl of substrate DNA, calf thymus DNA which had been produced to contain tritiated methyl groups at the O^6 position of guanine (4000-5000 dpm). Extract dilutions were made using 2 - 50µl of extract made up to 200µl using Buffer A; each test dilution was performed in triplicate. Incubation of substrate and extract was performed at 37°C for 90 minutes, conditions which allowed the repair process to go to completion. The reaction was then stopped by the addition of 300µl of DNA/EDTA. Precipitation of residual substrate DNA and digestion of any [^{3}H]-methylated protein in association with DNA was achieved by the addition of 200µl of CTAB and 10µl of proteinase K respectively, then vigorously mixing and incubating tests and controls at 37°C for 60 minutes, followed by centrifugation at 13,000xg for 20 min to pellet the DNA. [^{3}H]-methyl groups associated with the enzyme were determined in a 650µl sample of the resulting supernatant, by its mixture with 4mls of Pico-Aqua scintillation cocktail (Canberra-Packard) and measurement of radioactivity in a scintillation counter (Canberra-Packard 2500TR). An enzyme dilution curve, prepared using a fixed amount of substate, was constructed for each extract. The molar amount of enzyme was then calculated from the linear portion of the curve using knowledge of the specific radioactivity of the [^{3}H]-methylated substrate (17.9 Ci/mmol or 40 x 10^3 dpm/pmol) and the stoichiometric nature of this DNA repair reaction i.e. one enzyme molecule reacts with one substrate molecule, and the enzyme has one active site (Figure 3.2.1).

DNA and protein estimation

Protein content of tissue extracts was determined by the Coomassie G-250 dye-binding method of Bradford using a BioRad protein assay kit and bovine serum albumin (Sigma) as the calibrating protein (Bradford, 1976). DNA was measured by fluorescence using a TK-100 fluorometer (Hoefer Scientific Instruments), Hoechst dye 33258 at 0.1µg/ml and calf thymus DNA as the standard (Cesarone et al, 1979). O^6-MT levels were expressed in two ways: amount per total extract DNA and per total extract protein.

3.2.2 Assay of O^6-MT in liver and lymphocytes

Case material

Forty eight patients admitted to Medical Unit 1, Freeman Hospital, Newcastle upon Tyne, for investigation and treatment of liver disease, were studied. All diagnoses were based on a combination of clinical, biochemical, serological and histological findings. Patients were classified as either having cirrhosis (n=23), non-cirrhotic liver disease (n= 19) or normal liver histology (n= 6); (Table 3.2.2). Those patients with normal liver histology had abnormalities in liver function tests prior to biopsy which subsequently returned to normal. Liver biopsy material upon collection was immediately snap- frozen in liquid nitrogen and stored at -80°C. Whole blood, for lymphocyte isolation, was taken from 30 patients at the same time as liver biopsy and from a further 11 healthy controls who had not had liver biopsies.

Isolation of lymphocytes and enzyme extraction

20mls of whole blood was collected into heparinised tubes. Isolation of lymphocytes was performed

A

O^6-MT

+

$[^3H]$ - CH_3- DNA
(substrate)

+ 37°C for 90 min

SCH_3 - $[^3H]$

Methylated
tritiated
O^6- MT

+

DNA

+ CTAB/proteinase K

DNA precipitated

$[^3H]$ enzyme measured in a scintillation counter

B

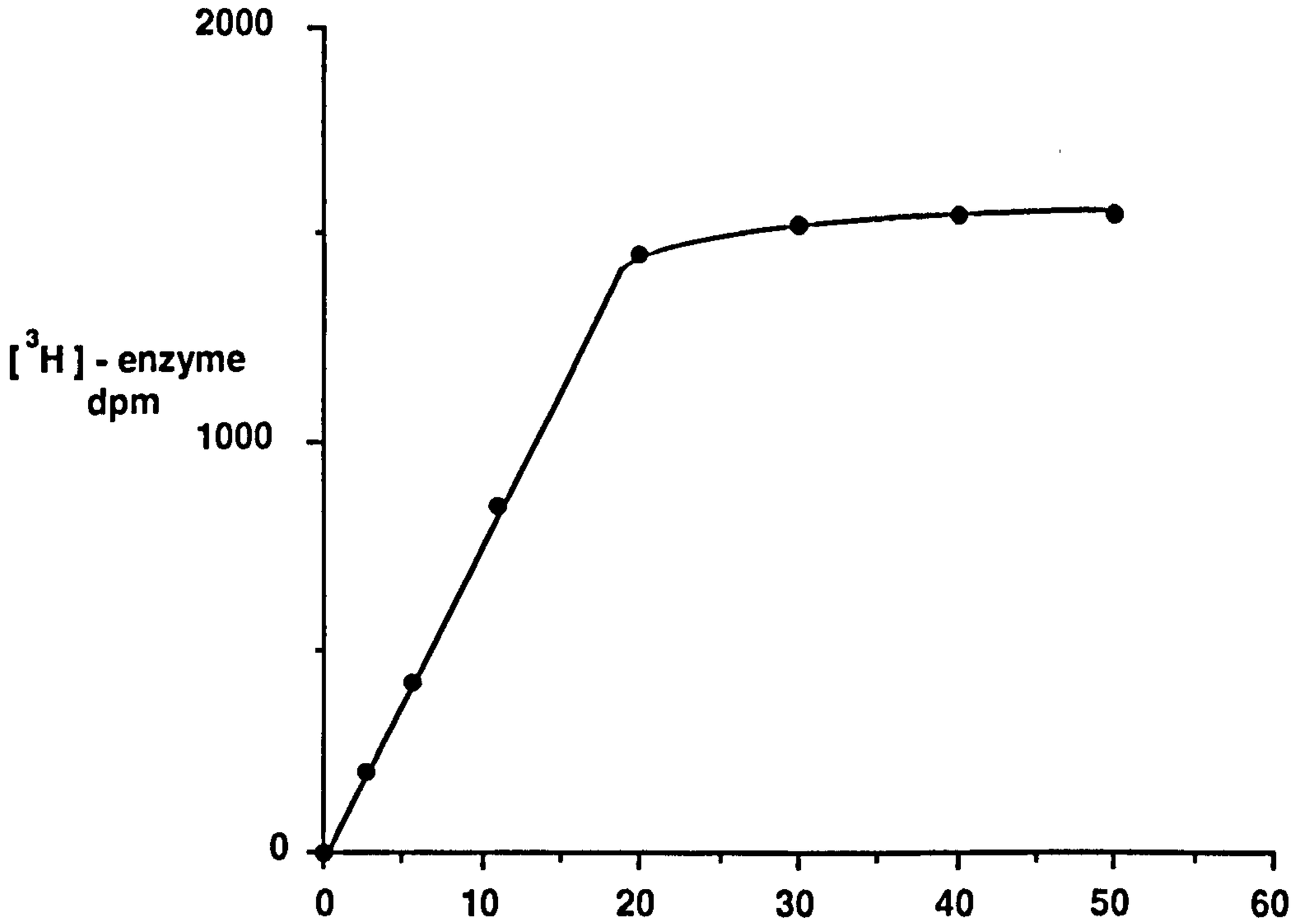


Figure 3.2.1: Enzyme assay. (A) Schematic representation of the enzyme assay illustrating the stochiometric reaction between O^6-MT and methylated DNA substrate. The SH group represents the cysteine residue at the active site of the enzyme. (B) Typical enzyme dilution curve showing radiolabelled enzyme in the extract as a function of extract volume following reaction with a fixed amount of substrate; at higher extract volumes the substrate concentration becomes limiting. Activity is derived from the linear portion of the curve.

Table 3.2.2: Clinical details on 48 patients from whom liver biopsy tissue was available from for O^6-MT assay.

	Cirrhosis	Non-cirrhotic liver disease	Normal
n	23	19	6
Age (yrs)*	52 (23-75)	53 (24-72)	51 (30-62)
Male: Female	14:9	14:5	4:2
Diagnosis	Alcoholic (n=11)	Alcoholic steatosis (n=13)	
	Cryptogenic (n=5)	Idiopathic steatosis (n=6)	
	Chronic active hepatitis (n=1)		
	Hepatitis C (n=4)		
	Primary biliary cirrhosis (n=2)		

* mean (range)

by density-gradient centrifugation through Lymphoprep (Nycomed AS, Oslo) according to the manufacturer's instructions. Briefly, 10mls of whole blood was layered onto 6 mls of Lymphoprep and centrifuged at 15000 rpm for 20 minutes. The lymphocyte band was carefully removed using a glass pasteur pipette and washed by centrifugation at 1800 rpm for 15 minutes in 10mls of phosphate buffered saline (PBS), pH 7.4 (Sigma). The pelleted lymphocytes were similarly washed again but using a 5 minute centrifugation time. This pellet was resuspended in 6 mls of PBS and a 10µl sample was removed for lymphocyte counting using a haemocytometer. Finally, the lymphocytes were pelleted by centrifugation at 1800 rpm for a further 5 minutes, the supernatant removed and the pellets stored frozen at -80°C.

Lymphocyte extracts were prepared in the same way as liver extracts except that homogenisation was performed in 600µl of Buffer A made 50mM with NaCl (Buffer B) to achieve extraction efficiency equivalent to that obtained with liver tissue (personal communication; Dr K Guo, Medical Molecular Biology Group, University of Newcastle upon Tyne).

Statistics

Analysis of variance (ANOVA; *F*-test) was used to compare differences in tissue O^6-MT levels between the 3 different groups of patients. Mean enzyme levels were compared between cirrhotic patients and either non-cirrhotic liver disease patients or individuals with normal liver histology, using a *t*-test. Lymphocyte and liver levels of O^6-MT from the same patients were compared by linear regression analysis. Calculations were made using "Statview 512+" software on an Apple Macintosh computer.

3.3 Results

3.3.1 Preparation and storage of human tissue and cell extracts containing O^6-MT

Human liver sample storage

No O^6-MT was present in post mortem liver biopsies, suggesting that the enzyme may have been inactivated as a result of proteolytic digestion. Optimal tissue storage, therefore, necessitated immediate snap-freezing in liquid nitrogen and storage at -80°C. In this manner liver could be stored for 1 year without appreciable loss of activity. Normal liver which had been subjected to frequent episodes of thawing and refreezing had significantly lower levels of O^6-MT than optimally stored tissue (mean±SEM fmol/mg protein of 649±40 vs 2322±110; p= 0.0001) as did tissue stored for a prolonged period (5 years) at -80°C (573±46 vs 2322±110; p= 0.0001). The inter-assay precision (expressed as a coefficient of variation) for optimally stored tissue was 11%, compared to that for liver stored for a prolonged period of 30% and 24% for liver thawed repeatedly (Figure 3.3.1a).

Liver sample handling and enzyme extraction

Following removal from -80°C storage weighing of tissue at room temperature prior to enzyme extraction resulted in loss of O^6-MT activity. Consequently, liver biopsy tissue could not be routinely weighed, and enzyme levels are expressed in relation to measured tissue protein and DNA content, but not wet weight of tissue. However the wet weight of normal liver did

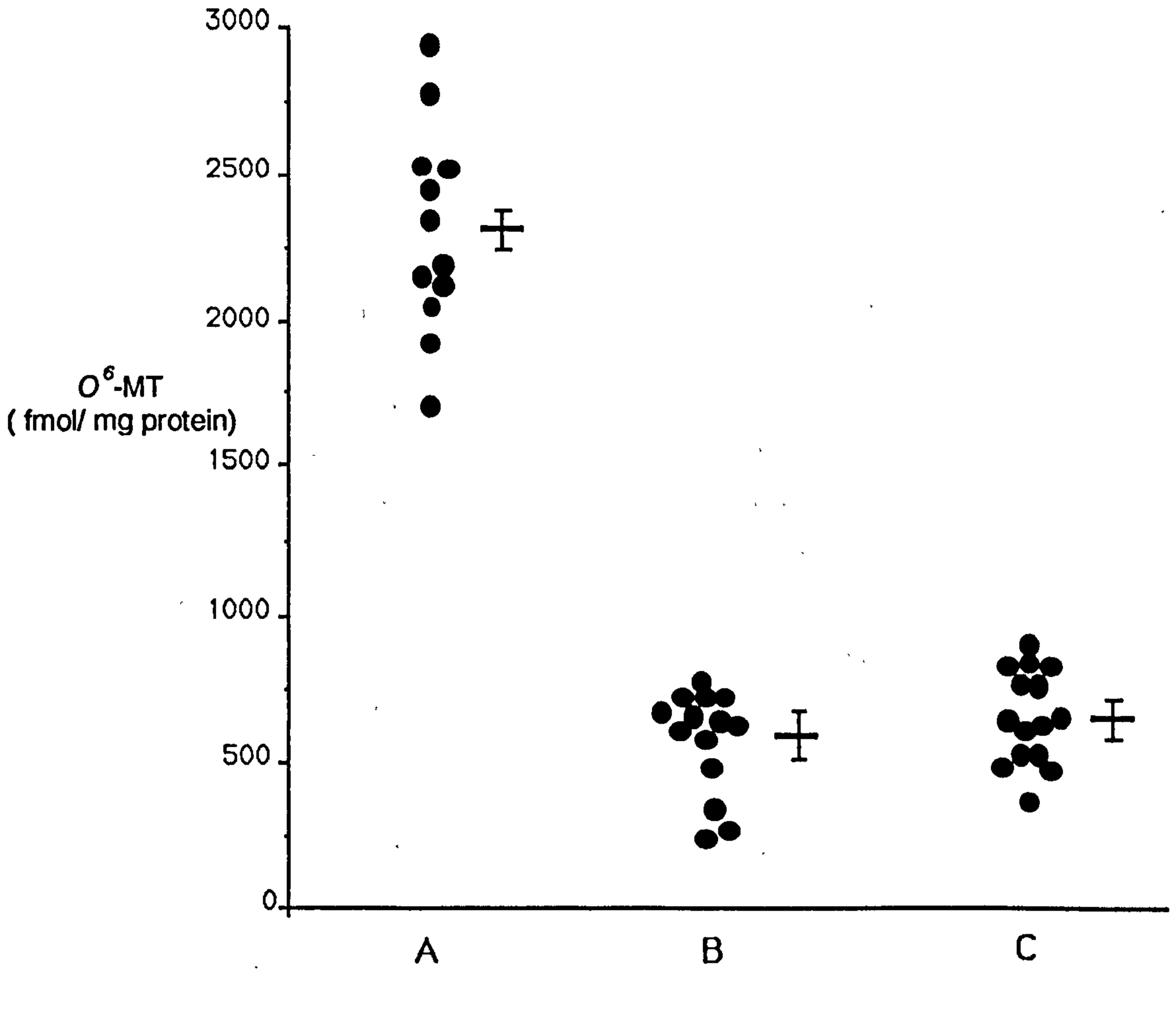


Figure 3.3.1a: O^6 - MT levels measured in extracts prepared from normal liver stored at (A) -80°C for less than 1 year, (B) at -80°C for 5 years, (C) defrosted/refrozen repeatedly prior to extraction and enzyme assay. Mean level (±SEM) of multiple biopsies from same liver sample, assayed on separate days is indicated by the horizontal bars. The mean (±SD) in fmol/mg protein was (A) 2322 (±365), (B) 573 (±173) and (C) 649 (± 157).

correlate with protein in liver extracts (n = 12 ; r= 0.921; p=0.0001), the mean being 71 mg protein /gram wet tissue. This value is similar to that previously reported by others for normal liver (Bielicki et al, 1990 ; Tuchman et al, 1990) and indicates that extracted protein levels reflect the amount of tissue used to produce the extract despite the fact that the protein content only accounts for about 7% of tissue weight.

Effect of storage of extracts prior to assay on enzyme levels

Incubation of liver extracts at increasing temperatures in the range of 0 to 37°C for up to 5 hours prior to enzyme assay resulted in appreciable loss of O^6-MT which was progressive with time and temperature (Figure 3.3.1b). This finding is in accordance with Major et al who showed in human spleen extracts that while the enzyme was labile to high temperature this effect was not observed following the addition of substrate DNA which stabilised the enzyme (Major et al, 1991). Extract handling conditions that preserved enzyme activity prior to enzyme assay were found to be keeping extracts at 0°C on ice after their preparation, assaying for O^6-MT as soon as possible , then storing remaining extract at -80°C. Prolonged storage of these extracts at -80°C still resulted in loss of O^6-MT, with only 65% of the enzyme remaining at 2 months. Such enzyme instability was not, however, seen with human lymphocyte extracts prepared and stored in a similar manner (Table 3.3.1a).

Extraction efficiency

As O^6-MT is a DNA repair enzyme it can be expected to have a predominantly nuclear distribution. Tissue homogenates were, therefore, made under conditions which promote whole cell disruption; namely freeze/thawing, the use of hypotonic buffer and blending using a rotating metal blade. Under these conditions nuclear enzyme should pass into the supernatant. The efficiency of this process was confirmed by the finding of 90% of the enzyme, present in the homogenate, in the supernatant with only 10% in the residual pellet (Table 3.3.1b).

3.3.2 Variation in O^6-MT levels

With liver tissue stored at -80°C, extraction followed by immediate enzyme assay gave an intra-assay variation in O^6-MT levels of 10-14% (Table 3.3.2). The inter-assay variation, a measure of day to day variability, was 11%.

3.3.3 O^6-MT in liver biopsies and lymphocytes

O^6-MT levels were measured in liver biopsies from cirrhotic (n=23), non-cirrhotic diseased liver (n=19) and normal liver (n=6). There was a highly significant difference in enzyme levels expressed in relation to total extract protein, between the 3 groups of patients (F = 28; p = 0.0001). The mean level of O^6-MT was significantly lower in the cirrhotic group when compared to the non-cirrhotic liver disease (p = 0.0001) and normal groups (p = 0.0001). Lymphocyte levels of O^6-MT, however, did not differ significantly between any of the patient groups (F = 0.29; p = 0.75), and were generally higher than liver levels in all patient groups when expressed in relation to total extract protein and lower when expressed in relation to total extract DNA (Table 3.3.3).

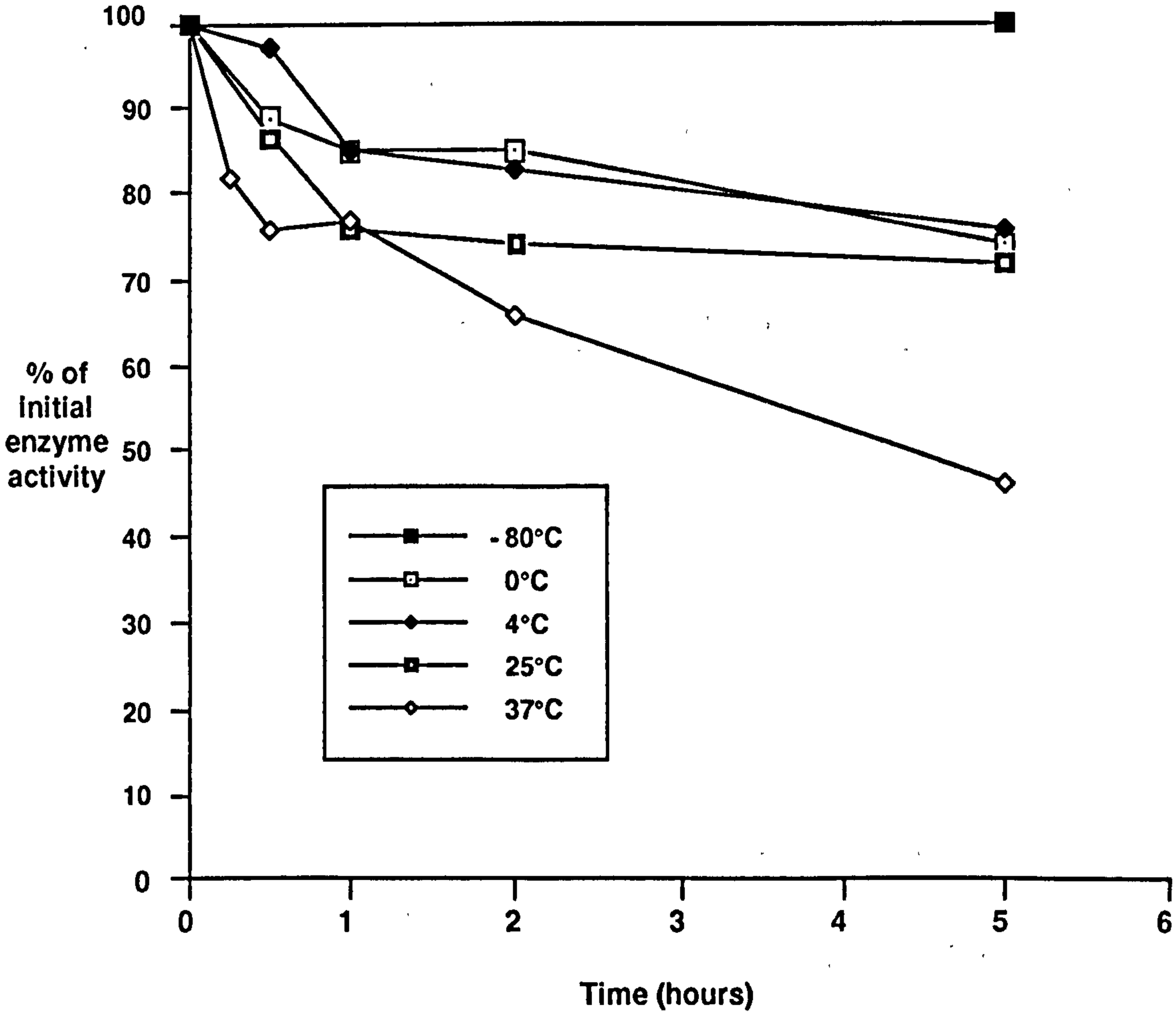


Figure 3.3.1.b: Effect of temperature on enzyme activity. Liver extracts were preincubated, immediately following enzyme extraction, at different temperatures for 0 - 5 hours prior to O^6 - MT assay. Enzyme levels were expressed as a percentage of activity present at time 0.

Table 3.3.1a: Effect of prolonged storage of normal human liver and lymphocyte extracts at -80°C on O^6-MT activity. O^6-MT was assayed in liver and lymphocyte extracts from two patients immediately following extract preparation (initial O^6-MT) and then following 2 to 8 week periods of storage.

Duration of extract storage at -80°C (Weeks)	% of initial O^6-MT			
	Liver extract		Lymphocyte extract	
	Patient 1	Patient 2	Patient 1	Patient 2
0	100	100	100	100
2	75	100	100	100
4	65	95	100	100
8	65	86	100	100

Table 3.3.1b: Efficiency of O^6-MT extraction from normal human liver on three consecutive days. O^6-MT was measured in the supernatant(extract) and residual pellet following centrifugation of the homogenate. The total amount of O^6-MT following cell disruption, reflected in the homogenate levels, is compared with the supernatant and residual pellet. The % of enzyme in the supernatant reflects the extraction efficiency.

Tissue fraction	O^6-MT (fmol)		
	Day 1	Day 2	Day 3
Homogenate	404 (100%)	828 (100%)	390 (100%)
Supernatant (extract)	375 (93%)	746 (90%)	341 (87%)
Residual Pellet	35 (7%)	86 (10%)	39 (10%)

Table 3.3.2: Intra-assay variation in liver extract O^6-MT levels prepared from normal liver on three consecutive days. Extracts were prepared from 4 samples from the same liver on each day. The intra-assay variation is expressed as a coefficient of variation.

Day	Number of samples	Mean (fmol/mg protein)	SD	SEM	Coefficient of variation
1	4	2643	261	131	10%
2	4	2111	294	148	14%
3	4	2235	293	146	13%

Table 3.3.3: O^6-MT in liver and lymphocyte extracts prepared from different patient groups. Enzyme levels from patients with cirrhosis, non-cirrhotic liver disease and normal liver histology are expressed as a mean(SEM) and related to both total cellular protein and total cellular DNA.

	Normal	Non-cirrhotic diseased liver	Cirrhotic
Liver O^6-MT	(n = 6)	(n = 19)	(n = 23)
fmol / µg DNA	989 (111)	870 (68)	540 (57)
fmol / mg protein	2164 (270)	1823 (75)	1016 (87)
Lymphocyte O^6-MT	(n = 15)	(n = 15)	(n = 11)
fmol / µg DNA	1138 (92)	1449 (128)	1193 (74)
fmol / mg protein	943 (71)	985 (105)	872 (137)

Levels of O^6-MT were determined both in relation to total extract DNA and total extract protein as the protein to DNA ratio differs between lymphocyte and liver extracts. With regard to solid tissue, DNA measurement will provide a more accurate reflection of cellularity thus enabling direct comparison with lymphocyte enzyme levels. A significant difference in liver O^6-MT levels expressed in relation to extract DNA was also evident between the 3 patient groups ($F = 9.91$; $p = 0.0003$), with mean levels of O^6-MT being significantly lower in cirrhotic tissue than either non-cirrhotic diseased liver ($p = 0.0006$) or normal liver ($p = 0.01$). Lymphocyte levels of O^6-MT expressed per extract DNA did not differ significantly different between the 3 groups ($F = 2.64$; $p = 0.085$).

When expressed in relation to total extract protein, there was a two- to three-fold inter-individual variation in O^6-MT levels in normal and non-cirrhotic liver, and a seven-fold variation in cirrhotic livers. A similar variation was also seen between the same groups (2, 3 and 6.5-fold, respectively) when O^6-MT was expressed in relation to total extract DNA (Figure 3.3.3). Lymphocyte levels in all three groups displayed three- to four-fold interindividual variation when expressed in relation to either total extract protein or DNA.

3.3.4 Relationship of O^6-MT levels between liver and lymphocyte extracts

Overall comparison of O^6-MT levels (expressed in relation to extract DNA) between matched lymphocytes and liver extracts prepared from all three patient groups revealed no significant correlation ($r = 0.259$, $p = 0.159$, $n=30$). This lack of correlation extended to the subgroups of patients considered separately; non-cirrhotic liver disease ($r = 0.35$, $p = 0.2$, $n=15$) and cirrhotic patients ($r = 0.27$, $p = 0.42$, $n=11$). This comparison could not be made with matched normal liver and lymphocytes due to low sample number ($n=4$). A similar lack of correlation was also observed when O^6-MT levels were expressed in relation to extractable protein(Figure 3.3.4).

3.4 Discussion

The feasibility of measuring O^6-MT in small gastric biopsies with an inter-assay precision of ±10% has previously been reported (Dyke et al, 1990). This study shows that O^6-MT can also be measured in human liver biopsies with a similar inter-assay variation of 11%. Instability of O^6-MT is well recognised but the hepatic enzyme appears to be particularly labile. Prolonged storage of liver tissue and extracts at -80°C resulted in enzyme loss a phenomenon which is not seen with lymphocyte pellets or extracts. It is therefore possible that part of the variation in liver enzyme levels reported in the literature (Table 3.1a) is an artefact due to differences in tissue handling with variable loss of enzyme activity occuring during the time from biopsy to enzyme assay. This is supported by the much smaller 2-3 fold inter-individual variation observed in normal liver in this study, when compared to previous studies, where enzyme handling conditions had been optimised.

Similar inter-individual variation also occurs in other normal tissues. Differences range from 14 fold in stomach (Dyke et al, 1990), 10 fold in the colon to 4 fold in T lymphocytes (Strauss, 1990) and the brain (Wiestler et al, 1984). The contributions made by genetic and environmental factors to this

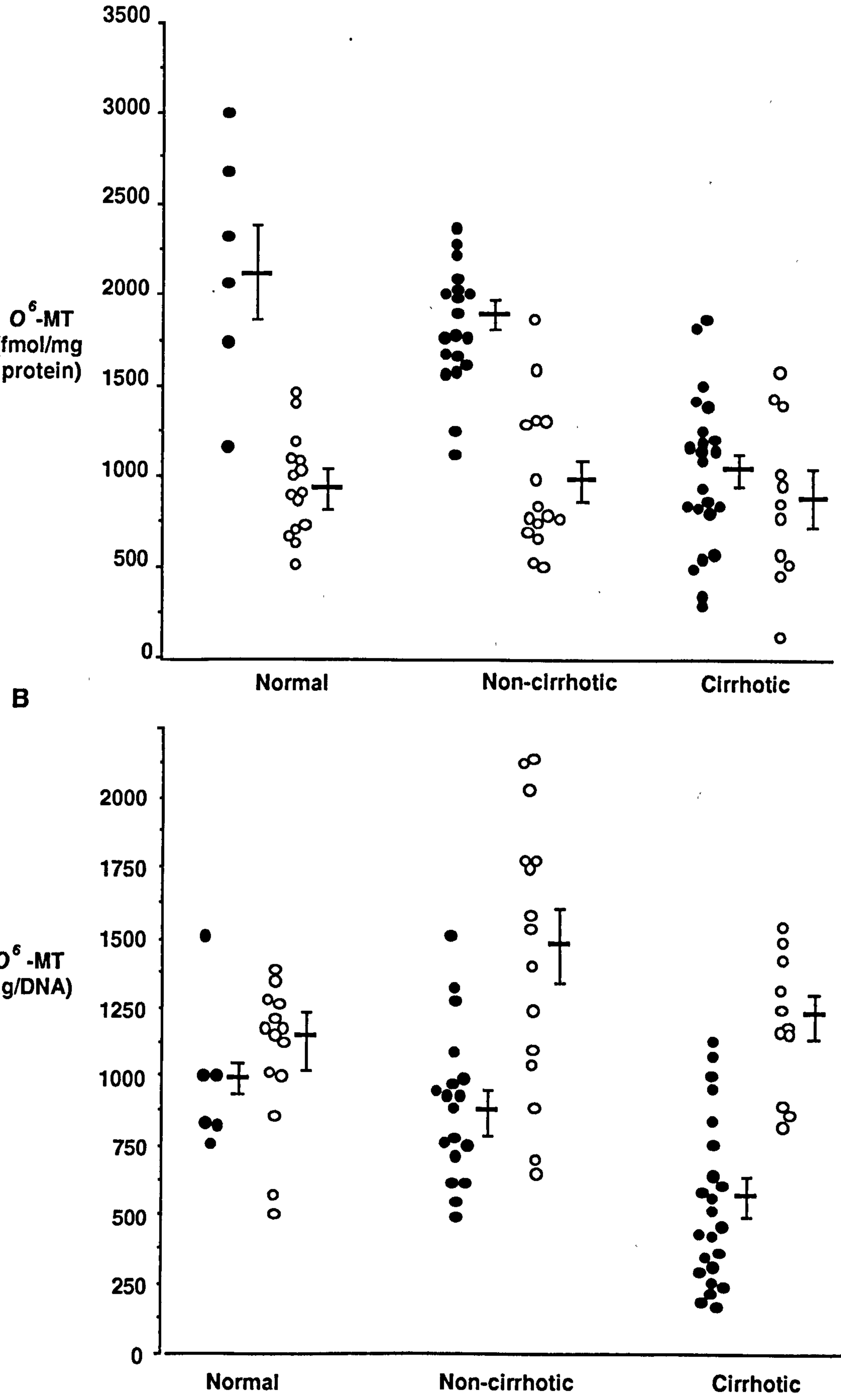


Figure 3.3.3: Liver and lymphocyte O^6-MT levels grouped according to underlying liver disease: normal, non-cirrhotic liver disease and cirrhotic liver.
Liver ●, lymphocytes ○. Enzyme levels related to (A) total extract protein and (B) total extract DNA. Horizontal bars represent the mean and vertical bars the SEM.

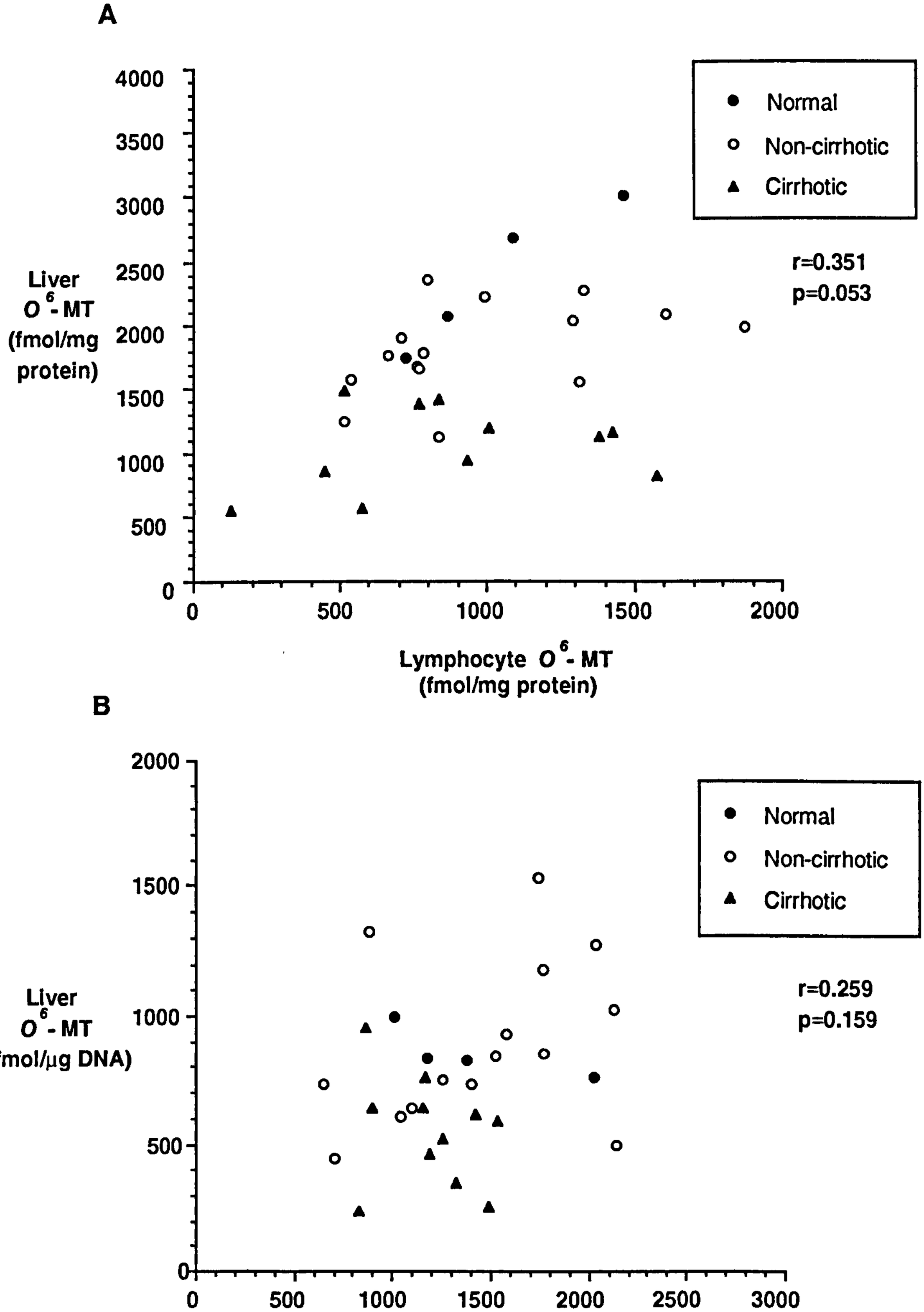


Figure 3.3.4: Lack of correlation between matched lymphocyte and liver O^6 - MT levels from individual patients with normal liver histology, non - cirrhotic liver disease and cirrhosis expressed (A) in relation to cellular protein, (B) in relation to cellular DNA.

variation has yet to be elucidated. Variations in O^6-MT levels within a tissue not only varies between individuals but also varies in the same individual with time. This cannot be quantitated in the liver because of the difficulties in obtaining multiple biopsy specimens. However lymphocyte levels measured 4 times over the course of a year differ by only 25% (intra-individual variation), much less than the 7 fold inter-individual variation seen in these cells (Sagher et al, 1988; Sagher et al 1989). Thus, if lymphocytes are representative of liver tissue intra-individual variation is unlikely to be a major contributory factor in liver tissue variability. The possibility that the 2-3 fold inter-individual variation seen in normal liver may be one factor determining the susceptibility of non-cirrhotic liver to the development of HCC is a hypothesis which deserves further evaluation.

Human susceptibility to carcinogens, whether acquired or inherited, appears to depend on several factors. From studies in animals susceptibility to nitrosamines might be expected to depend on the balance between 4 factors which together determine the extent of replication of alkylated DNA; (1) the ability of the tissue to metabolise nitrosamines to carcinogenic intermediates that alkylate DNA at the O^6 position of guanine, (2) the extent of adduct formation at the O^6 position of guanine, (3) the rate of removal of the O^6 alkyl adduct which will depend on the tissue level of O^6-MT and (4) the extent of DNA replication which occurs during a critical period when the adduct is present (Craddock, 1986). On this basis, cirrhotic liver tissue would be expected to be susceptible to nitrosamine-induced carcinogenesis; the liver is an important site for nitrosamine metabolism and cirrhosis is characterised by increased liver cell proliferation and DNA synthesis. The present finding of deficient repair of O^6-methylguanine in cirrhotic liver, as a result of lowered O^6-MT levels, provides a further reason for this tissue being at particular risk of malignant transformation following exposure to alkylating agents.

Although O^6-MT levels were significantly lower in cirrhotic livers as a group compared to non-cirrhotic livers there was a 7-fold inter-individual variation in enzyme within cirrhotic tissue. This variation was greater than that seen in normal liver and raises the possibility that a combination of both genetic and acquired factors determines O^6-MT levels in disease. The relatively large inter-individual variation of O^6-MT levels in cirrhotic tissue suggests that not all patients with cirrhosis will carry the same risk of developing hepatocellular carcinoma. This is consistent with cirrhosis of different aetiologies displaying a characteristic risk for the development of HCC (Bassendine, 1987). Larger studies are required to assess whether O^6-MT levels are lowest in those forms of cirrhosis associated with a higher risk of developing HCC, such as those due to haemochromatosis, chronic hepatitis B and C infection (Kew et al, 1990).

Epidemiological studies indicate that alcohol is associated with a 4-fold increased risk of developing HCC (Hardell et al, 1984). It is thought most likely to act as a co-carcinogen with other factors such as cirrhosis and hepatitis B infection (reviewed Bassendine, 1986). It is, therefore, of interest that both alcohol and its reactive metabolite acetaldehyde appear to inhibit both rat and human O^6-MT *in vitro* (Espina et al, 1988). Moreover, in rodents chronic alcohol consumption is associated with a reduction in liver O^6-MT levels (Garro et al, 1986). These studies have not been extended to alcoholic liver

disease in man. Although, in this study no difference in enzyme activity was seen between those with alcohol- and non-alcohol-related disease in either the non-cirrhotic or cirrhotic groups, a relationship between chronic alcohol abuse and impaired DNA repair cannot be excluded as the numbers in each group were small.

Old age and male sex are also important risk factors for HCC (Goodwin et al, 1986; Ihde et al, 1974). The effect of age on DNA repair by O^6-MT in normal human liver is unknown although fetal rodent liver has been shown to have lower levels of O^6-MT than adult tissue (Krokan et al, 1983). Once adult enzyme levels have been reached in rats there is then a progressive fall in activity with further increasing age (Pardini et al, 1992). Only one small study has attempted to correlate age with O^6-MT levels in man, and no correlation was seen between increasing age and enzyme levels in the 19 patients, over 75 years, studied (Edwards et al, 1989). In the light of the inter-individual variation in levels of this enzyme large population studies with repeated measurements of enzyme levels would be required to assess the effect of age on DNA repair by O^6-MT. Comprehensive studies looking at the effects of age, sex and aetiology of cirrhosis on O^6-MT levels in cirrhotic tissue are now feasible as a result of the development in this study of a reproducible method for the measurement of O^6-MT levels in liver biopsy samples.

The previously described correlation between gastric mucosa O^6-MT levels and levels in circulating lymphocytes raised the possibility that lymphocyte enzyme levels might serve as a surrogate marker of O^6-MT in less accessible tissues (Kyrtopoulos et al, 1990). If the same relationship existed between liver and lymphocytes studies designed to assess the contribution of hereditary factors to the setting of O^6-MT levels in chronic liver disease, by measuring lymphocyte enzyme levels in a large cohort of patients and their families would have been made feasible. Such a correlation, however, was not found between diseased liver and lymphocytes in the present study. It was not possible to determine whether such a relationship existed between normal liver and lymphocytes due to the small number of normal liver biopsies available. Nevertheless, the finding of no correlation between liver and lymphocytes indicates that the differences in O^6-MT levels seen between cirrhotic and non-cirrhotic liver are indeed disease- and target tissue-specific.

The finding of low levels of O^6-MT in cirrhotic tissue raises the question as to whether, at the cellular level, this deficiency is confined to hepatocytes, as opposed to non-parenchymal cells, and whether hepatocyte expression is heterogeneous. These are important questions as the susceptibility of cirrhotic tissue to carcinogenic damage may be expected to depend on the number of hepatocytes with low levels of O^6-MT rather than the overall tissue levels. These points could be answered by either using either *in situ* hybridisation to localise O^6-MT RNA or determining the intrahepatic distribution of the enzyme using immunohistochemistry .

3.5 Summary

Micro-assay For Measuring Liver O^6-MT

- Using a combination of a DNA repair substrate of high specific radioactivity and optimising procedures for liver sample collection, storage, and enzyme extraction and assay, reproducible levels of O^6-MT activity were able to be determined in small 5-15mg biopsy specimens.

- Inter-assay variation was 11%

DNA Repair By O^6-MT In Cirrhotic Tissue

- O^6-MT levels (mean±SEM) :

normal liver (n=6)	989±111 fmol/µg DNA
non-cirrhotic diseased liver (n=19)	870±68 fmol/µg DNA
cirrhotic (n=23)	540±57 fmol/µg DNA

- O^6-MT levels were significantly lower in cirrhotic liver than non-cirrhotic diseased liver (p=0.0006) or normal liver (p=0.01)

- no correlation between liver and lymphocyte levels in patients with non-cirrhotic or cirrhotic liver disease, implying that deficient repair in cirrhosis is disease- and tissue-specific.

Chapter 4

Production And Characterisation Of Polyclonal Antibodies To O^6-methylguanine-DNA methyltransferase

4.1 Introduction

Tissue levels of native O^6-MT can be reliably measured by direct enzyme assay, as described in Chapter 3, and can be taken as an indication of a the potential ability of a tissue to repair promutagenic DNA damage by environmental alkylating agents. However this provides a measurement of the tissue as a whole and takes no account of cellular heterogeneity in O^6-MT expression. Heterogeneity may occur between different cell types within the same tissue. In the rodent lung Clara cells have a lower activity than alveolar cells (Belinsky et al, 1988). Similar heterogeneity is described in human kidney (Wani et al, 1992) and in the white blood cells where T lymphocytes have the greatest activity (Waldstein et al, 1982).

These considerations are particularly relevant to liver tissue which comprises a heterogeneous population of cells; parenchymal cells (or hepatocytes) and sinusoidal cells (Kupffer cells, perisinusoidal cells, endothelial cells) and other non-parenchymal cells (such as portal fibroblasts). The parenchymal to non-parenchymal cell ratio varies between normal and cirrhotic liver, the latter having a higher non-parenchymal component. In rat liver, parenchymal cells contain 4- to 5-fold higher levels of O^6-MT than non-parenchymal cells (Swenberg et al, 1982). This observation has not yet been extrapolated to human liver. Nevertheless, it raises the possibility that the low levels of O^6-MT observed in cirrhotic liver may simply reflect the expansion of the non-parenchymal cell population when compared with normal liver. It is also important to know whether the deficiency observed in cirrhotic tissue occurs in all hepatocytes as it may be that the number of cells deficient in the enzyme is more important than the overall tissue levels in relation to increasing susceptibility to carcinogens and thus the risk of tumour development.

The full-length human O^6-MT cDNA was recently cloned in an O^6-MT deficient strain of *E.coli* using a system where bacteria containing the gene of interest were selected by their ability to grow in the presence of alkylating agents (Tano et al, 1990; Rydberg et al, 1990; Hayakawa et al, 1990). The protein sequence encoded by this cDNA contains 207 amino acids with a predicted M_r of 21,700. The cysteine alkyl acceptor is probably at residue 145 within the sequence **P C H R V** (residues 144-148) (Tano et al, 1990). Support for this suggestion comes from the fact that this sequence is homologous to that found in *E.coli*, yeast and rodent methyltransferases, and that amino acid substitutions of this cysteine results in complete loss of enzyme activity (Ling-ling et al, 1992).

The cloning of human O^6-MT cDNA has made it feasible to produce antibodies to different regions of the protein from the predicted amino acid sequence. The aim of this study was to raise and characterise polyclonal antibodies to different peptide regions of O^6-MT which could then be used to assess the tissue distribution of O^6-MT in order to assess differences between subpopulations of cells in normal and cirrhotic liver.

4.2 Materials and methods

4.2.1 Reagents

Casein blocking buffer	0.5% casein (Hammarsten grade, BDH), 0.9% NaCl, 10mM Tris/HCl (pH7.6), 0.02% thimerosal (Sigma)
DAB (3,3' diaminobenzidine)	0.1% DAB (Sigma), 137mM NaCl, 20mM Tris/HCl (pH7.6), 10mM imidazole (BDH)
Nickel-DAB reagent	137mM NaCl, 28mM nickel sulphate, 100mM sodium acetate, 10mM imidazole, 0.05% DAB (pH6.0)
SDS-PAGE running buffer	0.025M Tris/HCl (pH8.3), 0.192M glycine, 0.1% sodium dodecyl sulphate (SDS)
Tris-buffered saline (TBS)	20mM Tris/HCl (pH7.6), 137mM NaCl
Tris/glycine electroblotting buffer	0.2M Tris, 50mM glycine
SDS-PAGE sample buffer	4mls 10% SDS, 2mls glycerol (GIBCO BRL), 1ml 2-mercaptoethanol (Sigma), 2.5ml 0.5M Tris/HCl (pH6.8) 1.2ml 0.1% pyronin Y (Bio-Rad), 9.3mls H_2O

4.2.2 Peptide synthesis and conjugation

Peptides MDKDCEMKRTTLDSPLGKLE (N-terminal or P1), HEGHRLKPGLGGS (Internal site or P2), and KGAGATSGSPPAGRN (C-terminal or P3) were synthesised on an Applied Biosystems peptide synthesiser and their purity confirmed by high performance liquid chromatography, amino acid analysis and mass spectroscopy (Epitope Custom Peptides, Newcastle upon Tyne). A carboxyl terminal cysteine residue was added to P2 to facilitate hapten conjugation to keyhole limpet haemacyanin (KLH) carrier protein.

P1 and P2 were conjugated to KLH (Pierce) through their cysteine residues using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester according to the suppliers instructions (Pierce). P3 was conjugated to glutaraldehyde-activated-KLH as follows: 20mg of KLH (Imjet, Pierce) was dissolved in 2.5ml H_2O, and applied to a NAP-25 column desalting column (Pharmacia), equilibrated in 0.1M Na HCO_3 then eluted in 3.5ml of the latter. 5mg of P3 was dissolved in the eluate containing 5mg of KLH. This mixture was made up to a total volume of 1.5ml with 0.1M $NaHCO_3$, then mixed overnight at room temperature with 5µl of glutaraldehyde (EM grade, Sigma). P3-KLH conjugate was then, desalted/purified on a NAP-25 column, pre-equilibrated in PBS, according to the manufacturers instructions (Pharmacia) and eluted in a final volume of 3.5ml of PBS. The protein content of all eluates, containing purified conjugates, was measured by the Bradford method (Bradford, 1976) following sterile filtration through a 0.2µm membrane.

4.2.3 Immunisation

Half-Lop rabbits were pre-bled to provide pre-immune sera, then immunised by multiple subcutaneous injections of 100µg (P1 and P2) or 250µg (P3) of peptide emulsified with 1ml of Freund's complete adjuvent (Sigma)(Day O). The immunisation protocol was in keeping with Home Office Guidlines under an approved license. Two rabbits were immunised with each peptide. This was followed by a second immunisation 14 days later with the same amount of immunising conjugate mixed with 1ml of Freund's incomplete adjuvent (Sigma). Antibody production was monitored by enzyme-linked immunosorbent assay (see below) on serum taken 7 days following a boost. Further immunisations with P2 and P3, were carried out on days 28, 42, 56 and 84. Immunisations with P1 were carried out on days 28, 42, 56, 70, and 84. Rabbits were bled out on day 105. Whole blood was allowed to clot at 4°C overnight, then serum was prepared by centrifugation at 2000 rpm for 10 minutes, and stored in aliquots at -20°C.

4.2.4 Enzyme- linked immunosorbent assay (ELISA)

Antigen (pure peptide) was bound to 96-well microtitre plates (Immulon 4, Dynatech) by overnight incubation at 4°C with 100µl of appropriate peptide (P1, P2 or P3) at a concentration of 1µg/ml in each well. Unbound peptide was aspirated from the wells which were then washed three times with 200µl of casein blocking buffer. Unreacted sites in the wells were then blocked by incubation with 150µl of casein buffer for 1 hour at 37°C. Serial dilutions of immune sera, in the range of 10^{-2} to 10^{-6}, were then added in 100µl aliquots to the wells and incubated at 37°C for 60 minutes. The wells were emptied and washed as above, then incubated with 100µl of biotinylated goat anti-rabbit antibody (Amersham) diluted 1/1000 in casein blocking buffer for 1 hour at 37°C. Wells were aspirated and washed, then incubated with 100µl of streptavidin-biotinylated horse radish peroxidase (HRP) pre-formed complexes (Amersham) diluted 1/1000 in casein blocking buffer for 30 minutes at 37°C. Following a final aspiration and wash, detection of HRP bound to the wells was accomplished by adding 100µl of TMB reagent (Bio-Rad) per well. The enzyme reaction was stopped by the addition of 50µl of 2M H_2SO_4 (BDH) to each well after 1 minute. The absorbance in each well was measured at 405nm using a Titertek Multiscan MCC/340 ELISA plate reader.

4.2.5 Antibody purification

Anti-peptide antibodies were purified from crude rabbit sera by affinity chromatography on columns of immobilised peptide.

Peptide conjugation to BSA

P1 and P2 were coupled to BSA as follows: 2mg of peptide was mixed with 200µl of PBS and immediately with 2mg of Imjet maleimide-activated BSA (Pierce) dissolved in 200µl H_2O. The mixture was incubated for 2 hrs at room temperature and then made to 1ml with PBS. The conjugated peptide was purified by passage through a NAP-10 desalting column (Pharmacia), equilibrated in PBS according to the manufacturer's instructions. The protein content of the peptide

conjugate was measured as described by Bradford(1974).

P3 was conjugated to glutaraldehyde activated BSA as follows. 20mg of BSA (Imjet BSA, Pierce) was dissolved in 2.5ml H_2O, and purified by passage through a NAP-25 column, equilibrated and eluted with 0.1M Na HCO_3. 5mg of P3 was dissolved in eluate containing 5mg of BSA. This mixture was made to a total volume of 1.5ml with 0.1M $NaHCO_3$, then 5µl of glutaraldehyde (EM grade, Sigma) was added and the mixture left to react overnight at room temperature on a rotating mixer. Conjugated P3 was then purified on a NAP-25 column equilibrated in PBS.

Coupling of peptide -BSA conjugates to Sepharose-B

375mg of cyanogen bromide-activated Sepharose-B (Pharmacia) beads were washed and suspended in 5ml of 0.5M sodium phosphate buffer (pH7.5) to which was added 5mg of peptide-BSA conjugate. This mixture was left to react overnight at room temperature. The extent of conjugate bound to Sepharose was 57% for P1, 94% for P2 and 42% for P3. The beads were then washed twice with 0.5M sodium phosphate (pH7.5) followed by 1M NaCl, 0.05M sodium phosphate (pH7.5). Unreacted sites on the cyanogen bromide activated Sepharose beads were quenched by overnight incubation at room temperature with 10 volumes of 100mM ethanolamine (pH 7.5) and gentle mixing. The beads were then gently washed twice in PBS and stored at 4°C after addition of thimerosal (Sigma) to a final concentration of 0.01%.

Antibody purification by peptide affinity chromatography

The antigen-bound cyanogen bromide-activated sepharose beads were transferred to a 1ml column. Crude antisera was affinity purified as described by Harlow and Lane (1988a) using the relevant peptide affinity column.

Concentration of affinity-purified antibodies on columns of protein A - Sepharose

Following antibody purification by peptide affinity chromatography, antibody fractions were concentrated using protein A-Sepharose (Harlow and Lane, 1988b). The eluted antibody fractions were assayed for total protein using the method of Bradford (1974). The antibody was localised to fractions 2-4 which were pooled and stored in 0.02% sodium azide at 4°C. The yield of purified IgG from immunised rabbits following affinity purification was of the order of 200-400µg/ml. Pre-immune sera was either purified by (a) protein A-Sepharose chromatography or (b) by immunopurification through a relevant peptide affinity column followed by protein A-Sepharose concentration as described above.

4.2.6 Recombinant O^6-MT and cell lines

A small amount of recombinant O^6-MT, a generous gift of Dr P.Moody, Protein Structural Research Laboratories, Department of Chemistry, University of York, was available for use in the study and had an activity of 324 fmol/µl as supplied. Two cell lines were used: HT29, derived from a colorectal carcinoma and known to overexpress O^6-MT (Day et al,1980) and VA13, derived from an SV40-transformed human embryonic lung cell line, which has barely detectable levels of O^6-MT protein or mRNA (Day et al,1980; Wang et al, 1992).

4.2.7 SDS-PAGE and gel electroblotting

SDS-PAGE was performed using a discontinuous buffer system according to the method of Laemmli (1970) using 1.5mm thick 15% non-gradient polyacrylamide mini gels (10cm x 8 cm).

Gel casting

The constitution of the "resolving" and "stacking" gels are shown in Table 4.2.7.

Table 4.2.7: Constitution of 15% polyacrylamide" resolving" gel and 4% polyacrylamide "stacking" gel. The volumes used were sufficient to pour 4 gels.

Solution	15% resolving gel (lower)	4% stacking gel (upper)
40% acrylamide : bisacrylamide 37.5 : 1 (Amresco)	15 ml	2.01 ml
10% sodium dodecyl sulphate (BDH)	400 µl	200 µl
0.5M Tris/HCl (pH 6.8)		5 ml
1.5M Tris/HCl (pH 8.8)	10 ml	
10% ammonium persulphate (Sigma)	200µl	100µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)(Sigma)	20 µl	8µl
H_2O	14.38 ml	12.67 ml

Gels were cast in a mini-gel multiple casting device (Hoeffer Scientific Instruments) following the manufacturer's instructions. Once made, the gels were either used immediately or stored at 4°C overnight wrapped in plastic film to prevent dehydration.

Sample preparation

Recombinant O^6-MT (rO^6-MT) was used in both in its native form and following methylation. rO^6-MT was methylated by incubation with [^{3}H]-Me-DNA under conditions of the O^6-MT assay (see Chapter 3) for 90 minutes at 37°C, then concentrated by methanol/chloroform precipitation (Wessel and Flugge, 1984) prior to SDS-PAGE. Sample concentration was done in this manner so that equal amounts of methylated and non-methylated enzyme could be analysed in parallel. Precipitated tests were resuspended in 20µl of sample buffer. Native rO^6-MT was mixed with BSA, 200µg per 100 fmol of enzyme, to prevent loss of protein during SDS-PAGE.

HT29 and VA13 cell extracts were made and assayed as described for lymphocytes in Chapter 3. The HT29 cell extract had an activity of 56 fmol/10µl or 1330 fmol/mg protein whilst the VA13 extract had undetectable levels of O^6-MT.

Electrophoresis and blotting

All samples were electrophoresed under reducing conditions as follows: prior to electrophoresis samples were heated with an equal amount of sample buffer at 95°C for 5 minutes, then cooled. Test samples (40µl maximal volume) and protein molecular weight markers (Pharmacia; low range) were

subjected to electrophoresis using a minigel electrophoretic system SE250 (Hoefer Scientific Instruments) at 20mA/gel for 60-90 minutes. Following electrophoresis proteins were transferred to nitrocellulose by electroblotting in Tris/glycine buffer for 20 hours at 170mA without cooling, according to the method of Towbin, using a TE22 Mighty Small Transphor wet tank (Hoefer Scientific Instruments) (Towbin et al, 1979).

4.2.8 Protein staining of gels and electroblots

Polyacrylamide gels were subjected to general protein staining by microwaving for 2 minutes in 0.125% Coomassie blue R-250 (Bio-Rad), 50% methanol, 10% acetic acid , and then destaining for 1 hour in 50% methanol, 10% acetic acid followed by overnight destaining in 7% acetic acid, 5% methanol. Protein molecular weight markers electroblotted on to nitrocellulose were detected by staining with 0.1% amido black, 45% methanol, 10% acetic acid for 15 minutes followed by destaining in 70% methanol, 2% acetic acid for 40 minutes. The membranes were then rinsed in water and air dried.

4.2.9 Immunoprobing of electroblots

Electroblots of SDS-PAGE gels were blocked with 5% BSA dissolved in 20mM Tris/HCl (pH8.2), 0.9% NaCl ("blocking buffer") for 2 hours and then cut into strips for probing with anti-peptide antibodies as follows. Strips were placed in the wells of a mini-incubation tray (BioRad) covered with 400µl of primary antibody, diluted in 5% gelatin (Amersham), 20mM Tris/HCl (pH8.2), 0.1% BSA, 0.9%NaCl, 1% goat serum, and incubated for 2 hours at room temperature. Membrane strips were then washed 3 times in 0.1% BSA, 20mM Tris/HCl (pH8.2), 0.9% NaCl and probed with biotinylated goat antirabbit -secondary antibody (Amersham) diluted 1/500 in "blocking buffer" for 20 minutes. Strips were washed as above then incubated with pre-formed complexes of streptavidin-biotinylated horse radish peroxidase (Amersham) diluted 1/1000 in "blocking buffer" for 20 minutes. After further washing the strips were rinsed in H_2O. Peroxidase associated with the membrane was detected using nickel-DAB as the enzyme substrate as follows. 10mls of nickel-DAB was mixed with H_2O_2 (added to a final concentration of 0.02%) then filtered and immediately applied to the membrane strips for 5 minutes. The reaction was stopped by washing in H_2O.

4.2.10 Immunocytochemistry

Preparation of cell lines.

HT29 and VA13 cell lines were grown near to confluency on coverslips in Minimal Essential Medium Eagle (Sigma). Coverslips were then removed and washed in PBS. Cells were fixed by soaking the coverslips in either 100% acetone, 100% methanol or 4% paraformaldehyde for 10 minutes, then washed in PBS and air dried. Coverslips were attached to slides using DPX mountant (BDH) and stored at -20°C prior to use for immunostaining.

Immunostaining

Slides which had been stored at -20°C were allowed to warm to room temperature and then equilibrated in TBS. Protease digestion was achieved by a 10 minute incubation in 0.1% saponin. Non-specific binding was then blocked by incubating the slides with 3% normal swine serum (NSS) in TBS for 10 minutes. Coverslips were then covered with 100µl of primary antibody, diluted from 1/10 to 1/100 in TBS, or in 0.1% saponin in those cases where protease digestion had been undertaken, overnight at 4°C. Immunostaining was then detected using an indirect immunoperoxidase technique. Briefly, cells were washed in two 5 minute changes of TBS and incubated in 100µl of peroxidase -conjugated swine anti-rabbit immunoglobulin (Dako) at a diluted to 1/20 in NSS for 30 minutes. Following two further 5 minute washes in TBS cells were incubated with PAP (complex of horseradish peroxidase and rabbit anti-horseradish peroxidase) (Dako) diluted 1/50 in NSS for 30 minutes. Cells were again washed and immunolabelling was detected using filtered DAB containing 0.02% H_2O_2. The reaction was stopped by washing in water. Cells were counterstained with Mayers haematoxylin followed by Scott's tap water (Sigma), to enhance the nuclear counterstain, and mounted under coverslips using Glycergel (Dako).

4.2.11 Immunohistochemistry

5µm sections of normal human frozen liver, which had been embedded in OTC (Miles Inc, Elkhart, USA), and 3µm sections of paraffin-embedded normal liver tissue were attached to poly-L -lysine treated slides. The paraffin-embedded tissue used had been fixed in either 10% buffered formalin, 4% paraformaldehyde, or Bouin's solution. Paraffin-embedded sections were then dewaxed in xylene for 10 minutes and then rehydrated through graded alcohols (100% to 95% to 70%) to water. All sections were then equilibrated in TBS. Endogenous peroxidase was blocked by incubation in 0.5% hydrogen peroxide in methanol for 10 minutes. Where indicated some sections were protease-digested by incubation with 0.1% trypsin in 0.1% $CaCl_2$(pH7.8) for 10 minutes at 37°C. Non-specific antibody binding was blocked by pre-incubation of the sections with 3% normal swine serum (NSS). Anti-peptide antibodies against O^6-MT, that had been affinity -purified on columns of immobilised peptide, were used at dilutions between 1/10 to 1/100 in NSS. Incubations were performed using 100µl per section overnight at 4°C in moist incubation trays. Immunostaining was then performed using the indirect immunoperoxidase method followed by 3,3' diaminobenzidine as the chromagen as described above.

Sections which had been initially paraffin-embedded were then immersed in Lugols iodine for 3 minutes followed by 1% hypochlorite to remove any mercury present from the tissue fixative, and then counterstained with Mayers haematoxylin followed by Scott's tap water. Tissue sections were then dehydrated through 75%, 90% and 100% ethanol, immersed in xylene and mounted under coverslips using DPX mountant. Frozen liver sections were counterstained with Mayers haematoxylin followed by Scott's tap water and mounted under coverslips using Glycergel (Dako).

4.3 Results

4.3.1 Selection of peptides

Three peptides, peptides P1, P2 and P3, were predicted from the cDNA derived amino acid sequence (Tano et al, 1990) to be immunogenic based on hydrophilicity analysis (Hopp and Woods, 1981; Hopp, 1986; Kyte and Doolittle, 1982). These peptides contained amino acids 1-20, 171-184 and 193-207 of the predicted 207 amino acid sequence, and arise from the amino- and carboxy- termini regions and an internal site downstream from the highly conserved methyl acceptor site. The methyl acceptor site was not predicted to be antigenic (Figure 4.3.1).

4.3.2 Antibody production

All of the peptides usedproduced a good immune response as evidenced by the reaction of the rabbit antisera in ELISA against peptide-coated plates. Figures 4.3.2a, b and c show the rise in antibody titre in the 6 rabbits during the immunisation protocol. Antisera from rabbits A , D and F raised against peptides P1, P2 and P3 respectively gave the highest titres in ELISA and were used in immunoblotting experiments and immunocytochemistry.

4.3.3 Antibody specificity

The specificity of the antisera for O^6-MT was evaluated by testing their ability to detect recombinant O^6-MT following SDS-PAGE and electroblotting. Human recombinant O^6-MT as supplied was about 80% pure and was detected as a M_r 24,000 band when resolved by SDS-PAGE (Figure 4.3.3a). Although O^6-MT has a predicted M_r of 21,700 it exhibits slightly higher mobility by SDS-PAGE, giving a M_r of about 24,000; this is thought to be due to anomalous electrophoretic mobility of the enzyme (Koike et al, 1990). In preliminary studies variable amounts of rO^6-MT were detected on immunoblots following SDS-PAGE despite equivalent protein loading. This suggested that either the protein was not being carried from the "stacking" to the "resolving gel" during SDS-PAGE or the protein was not being transferred to membrane during electroblotting, and was probably a reflection of the small amount of protein used (less than 10ng). The problem was overcome by the the addition of a carrier protein, in the form of BSA, to rO^6-MT before SDS-PAGE resulting in increased transfer of the enzyme and thus a more intense M_r 24,000 band detected by immunoblotting as shown in Figure 4.3.3b. The M_r 57,000 band seen, when rO^6-MT was run with BSA, on the immunoblot in Figure 4.3.3b represents non-specific protein binding of the anti-N-terminal (anti-P1) antibody to a component of the BSA; this band is also seen in Figure 4.3.3d; lane 1.

All three affinity-purified anti-peptide antisera reacted with recombinant O^6-MT producing a band of M_r 24,000 on immunoblots (Figure 4.3.3c). Methylation of the enzyme did not appear to introduce a gross conformational change involving the three different epitopes recognised by the antibodies as all three antibodies reacted with methylated recombinant O^6-MT on immunoblots (Figure 4.3.3d). Antibody specificity was also tested by investigation of their ability to react with O^6-MT in extracts of

M D K D C E M K R T T L D S P L G K L E L S G C

1 10 20

E Q G L H E I K L L G K G T S A A D A V E V P A P

30 40

A A V L G G P E P L M Q C T A W L N A Y F H Q P

50 60 70

E A I E E F P V P A L H H P V F Q Q E S F T R Q V

80 90

L W K L L K V V K F G E V I S Y Q Q L A A L A G N

100 110 120

P K A T R A V G G A M R G N P V P I L I P C H R V

130 140

V C S S G A V G N Y S G G L A V K E W L L A H E

150 160 170

G H R L G K P G L G G S S G L A G A W L K G A

180 190

G A T S G S P P A G R N

200

Figure 4.3.1: Predicted amino acid sequence of O^6-MT, from cDNA, indicating the three peptides (underlined) used to raise polyclonal antibodies. P1: amino acids 1-20 (N-terminus), P2: amino acids 171-184, and P3: amino acids 193-207 (C-terminus). The cysteine methyl accepting site at the enzymes active centre is circled .

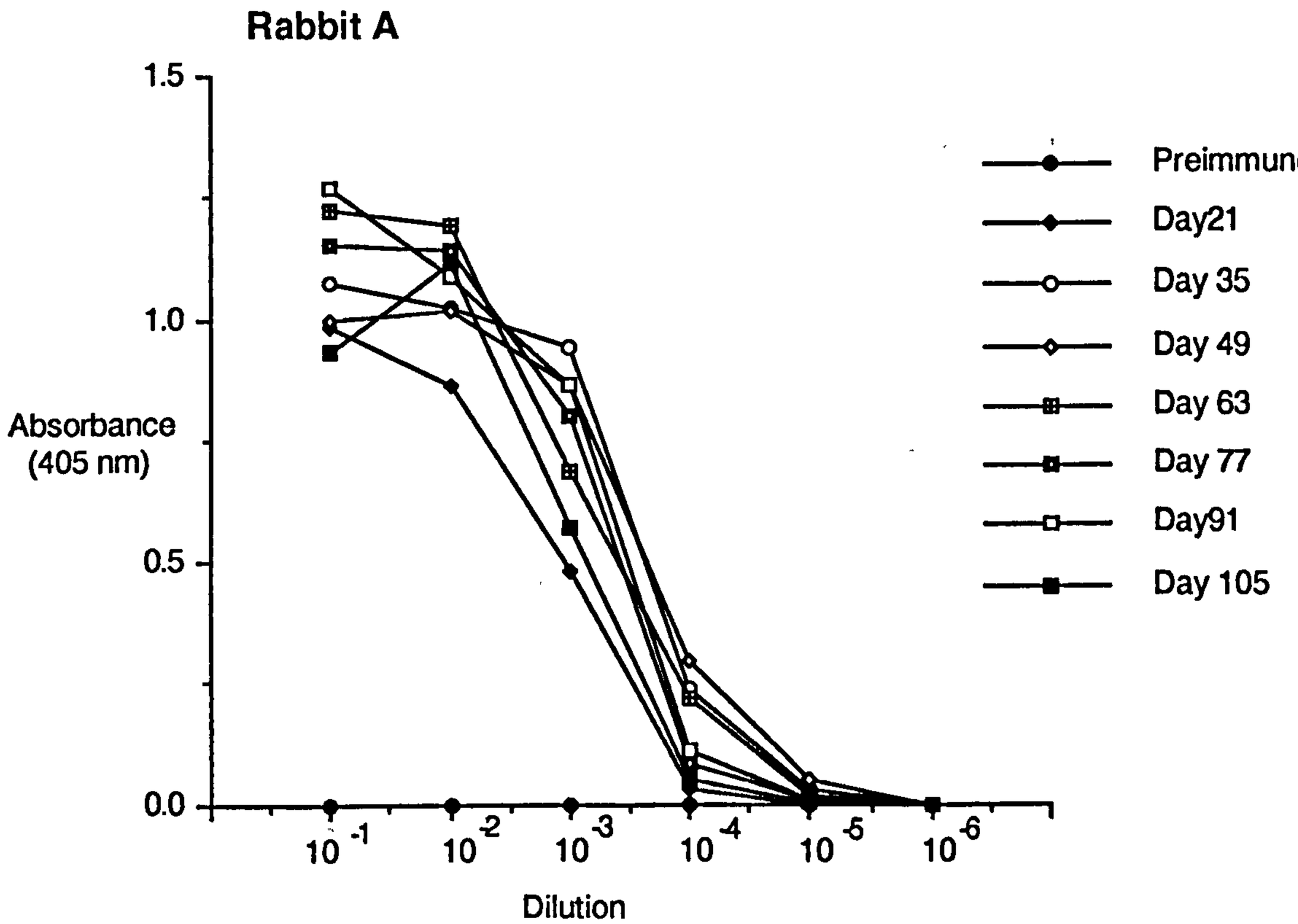
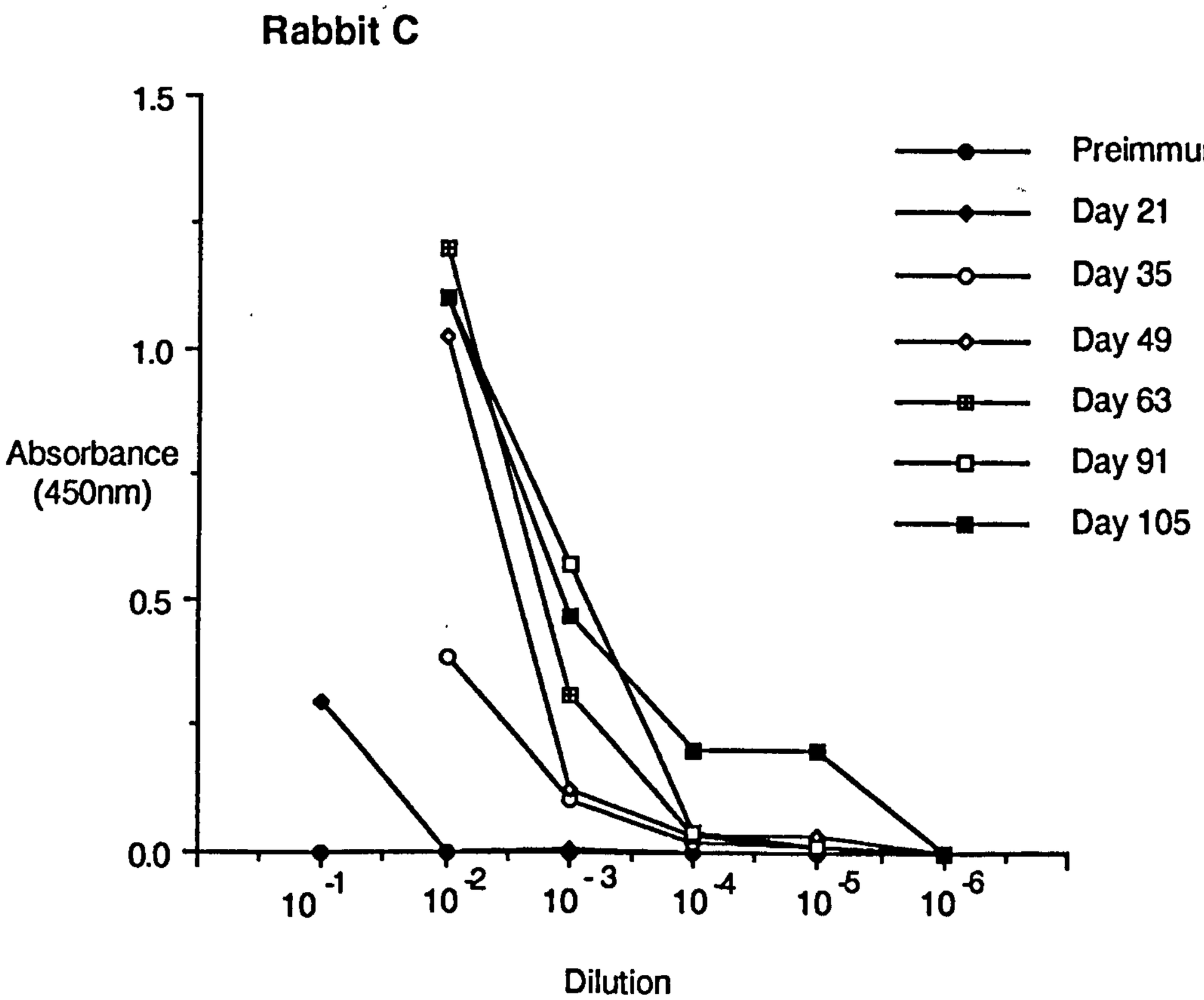


Figure 4.3.2a: Immunoreactivity determined by ELISA of rabbit antisera. Rabbits A and B were immunised with P1- KLH conjugate, and tested at the dilutions shown on days 0 through to 105 of the immunisation protocol.



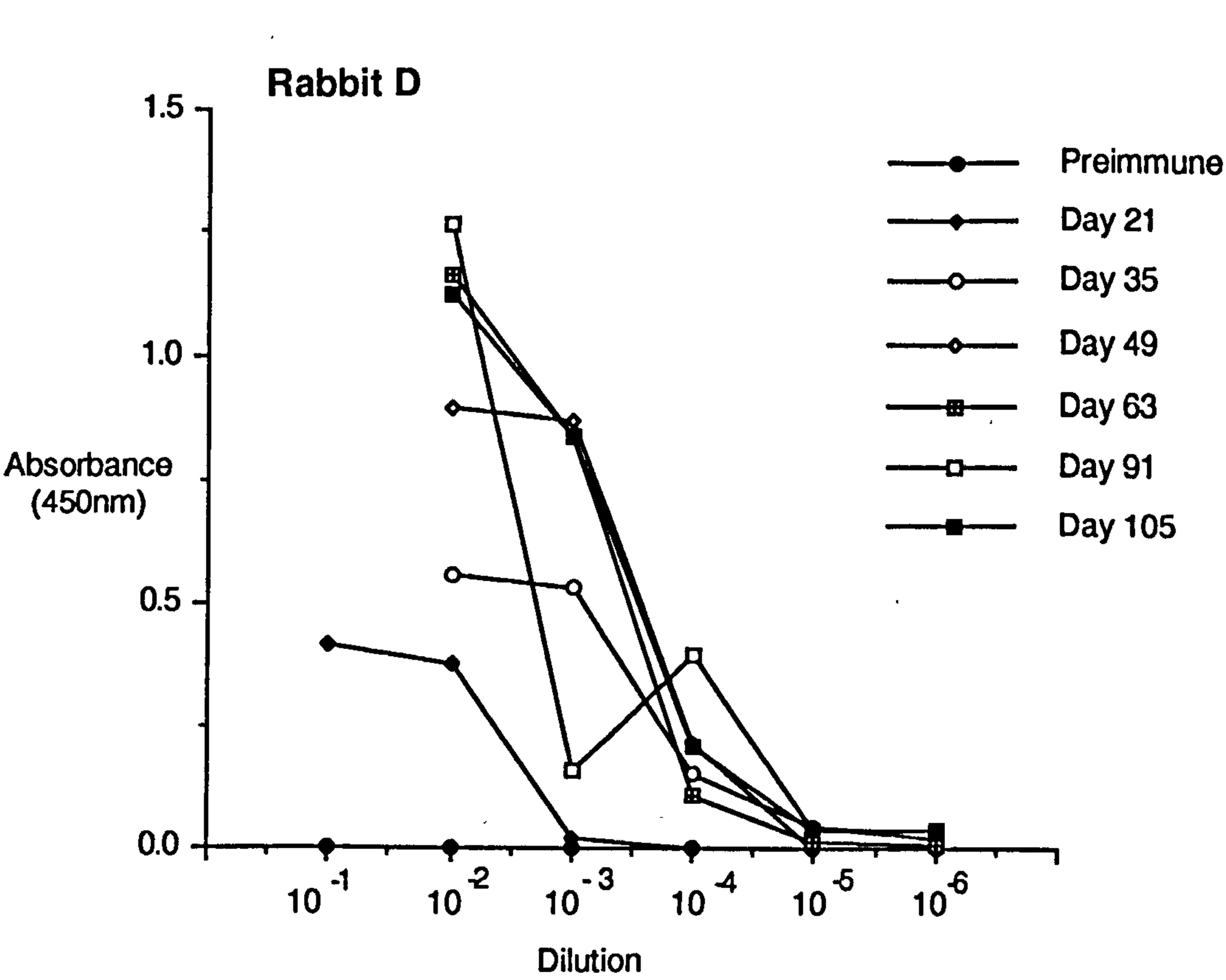


Figure 4.3.2b: Immunoreactivity determined by ELISA of rabbit antisera. Rabbits C and D were immunised with P2- KLH conjugate, and tested at the dilutions shown on days 0 through to 105 of the immunisation protocol.

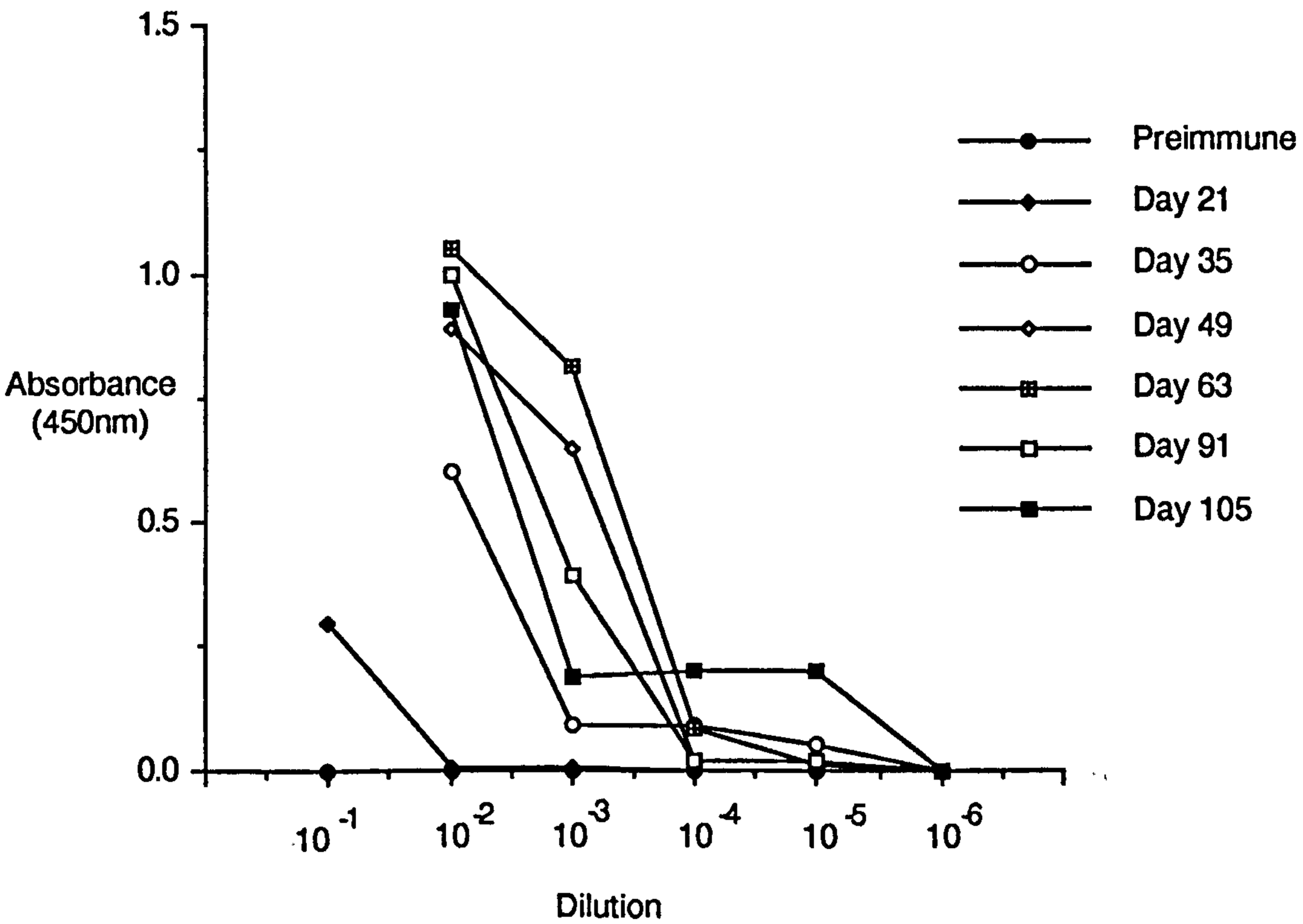


Figure 4.3.2c: Immunoreactivity determined by ELISA of rabbit antisera. Rabbits E and F were immunised with P3- KLH conjugate, and tested at the dilutions shown on days 0 through to 105 of the immunisation protocol.

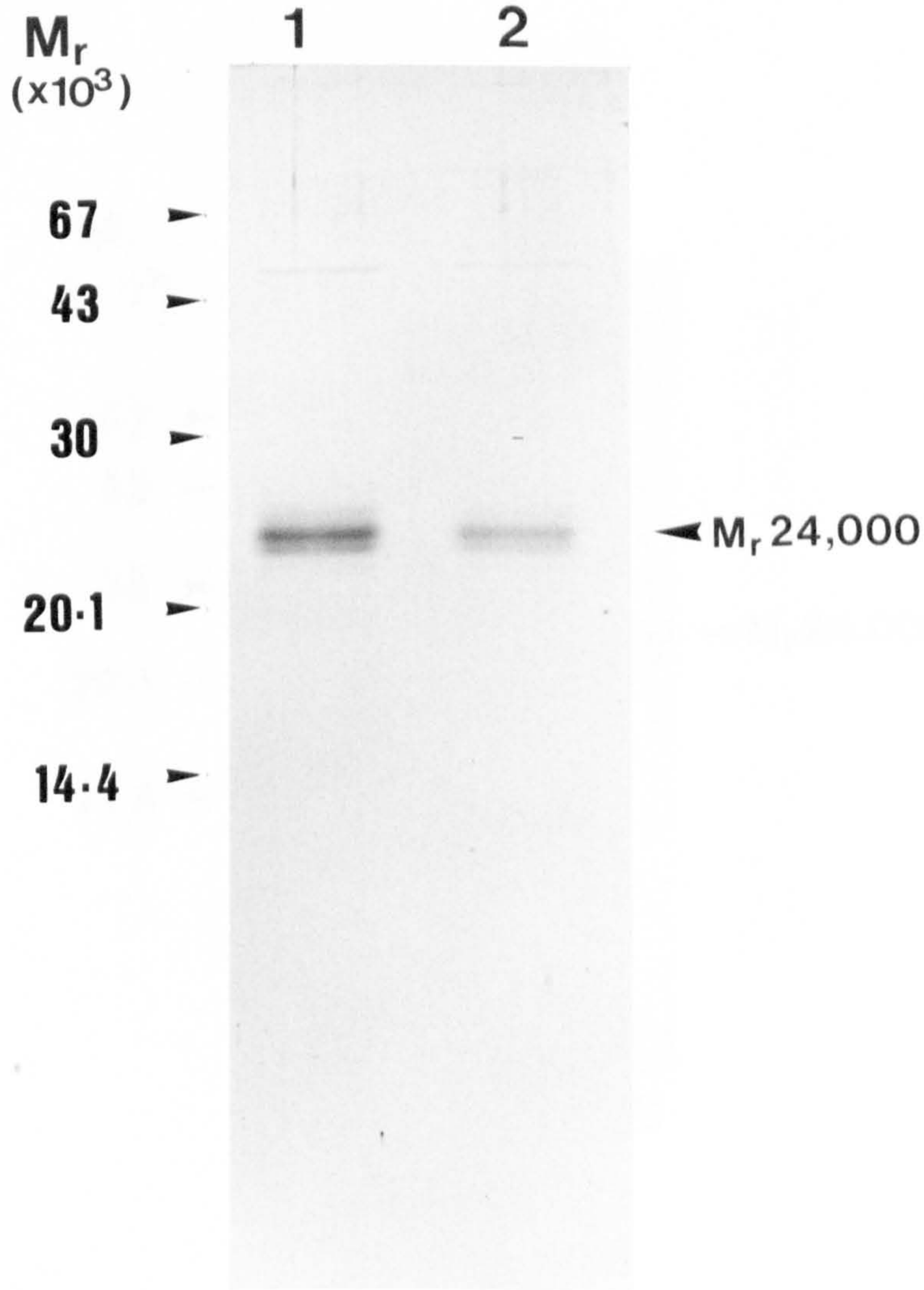


Figure 4.3.3a: SDS-PAGE of human recombinant O^6-MT (rO^6-MT). Coomassie blue stained 15% polyacrylamide gel. Lane 1: 70ng of rO^6-MT; lane 2: 35ng of rO^6-MT.

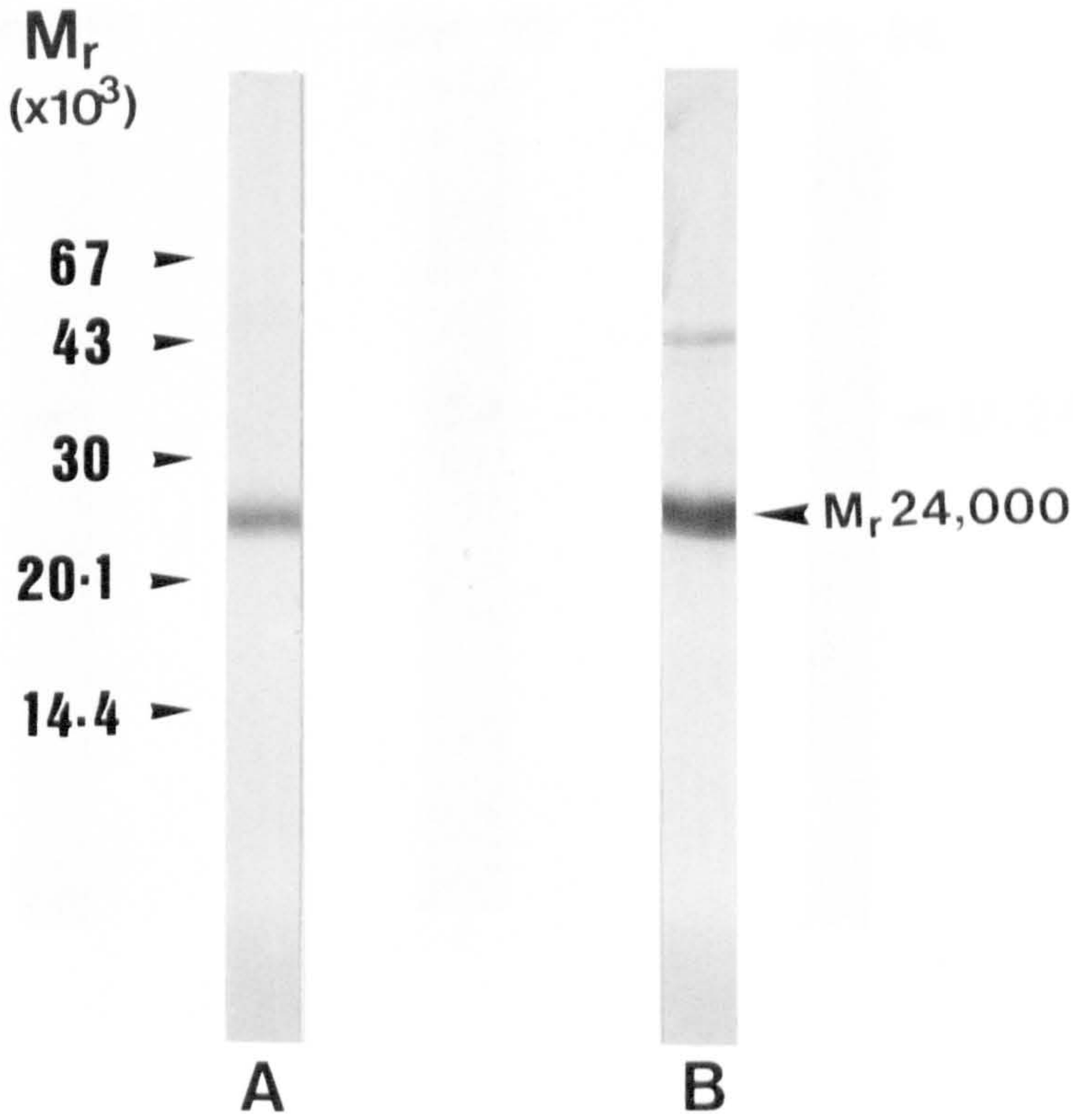


Figure 4.3.3b: Effect of adding carrier BSA to rO⁶-MT prior to SDS-PAGE. (A) 2.2ng of rO⁶-MT, (B) 2.2ng of rO⁶-MT + 25µg of BSA. Peptide affinity-purified anti-P1 (N-terminal) antibody was used to probe electroblot strips at a concentration of 3µg/ml.

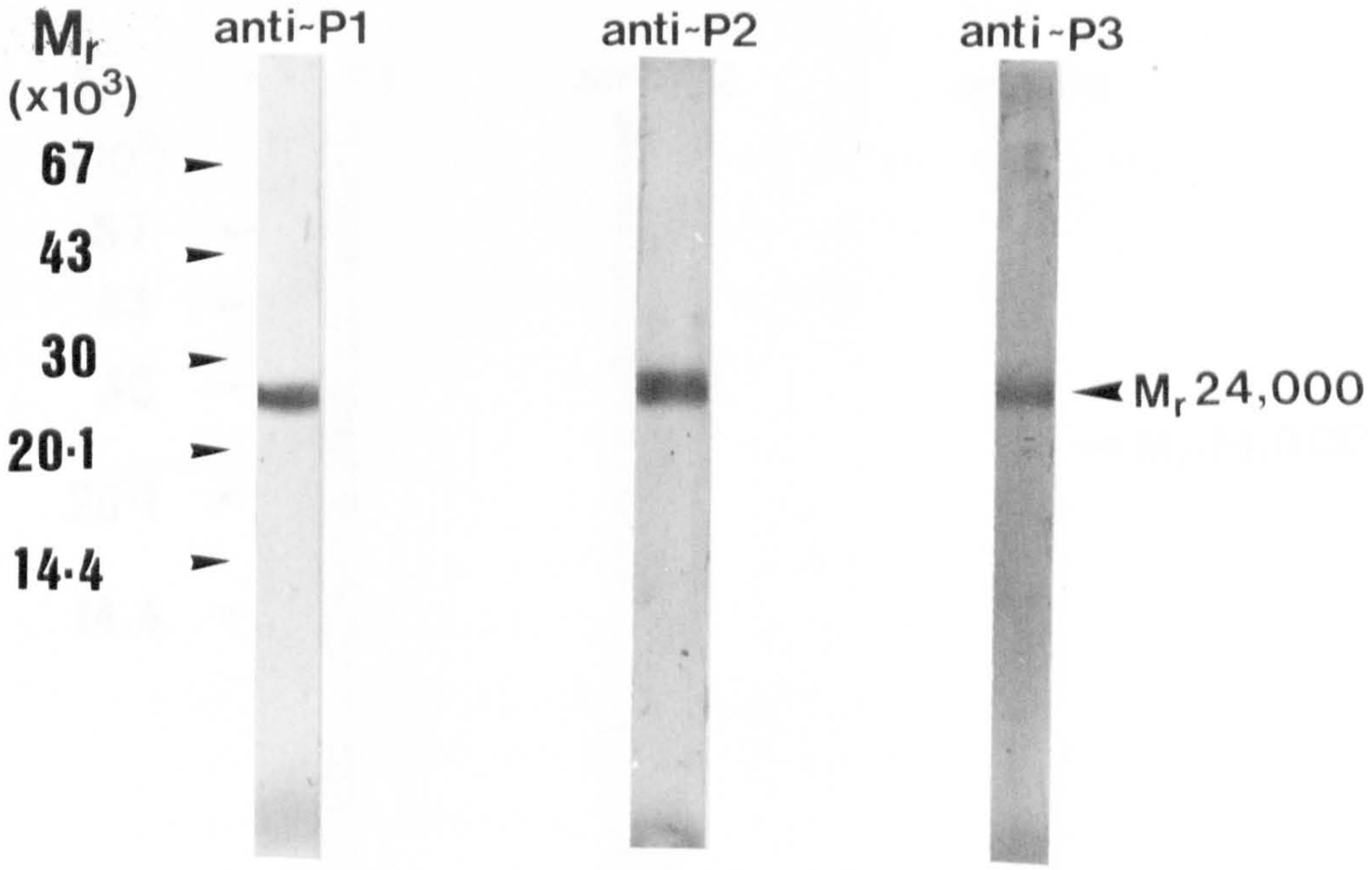


Figure 4.3.3c: Native (non-methylated/active) rO^6-MT detected on electroblot strips by the 3 different anti-peptide antibodies. Anti-P1 (directed against the N-terminus of the enzyme), anti-P2 (directed against an internal site) and anti-P3 (directed against the C-terminus) antibodies. 2.2ng of rO^6-MT in each lane was subjected to SDS-PAGE and electroblot strips were probed with anti-P1 (3µg/ml), anti-P2 (7µg/ml), and anti-P3 (3µg/ml) antibodies (all affinity-purified on peptide columns).

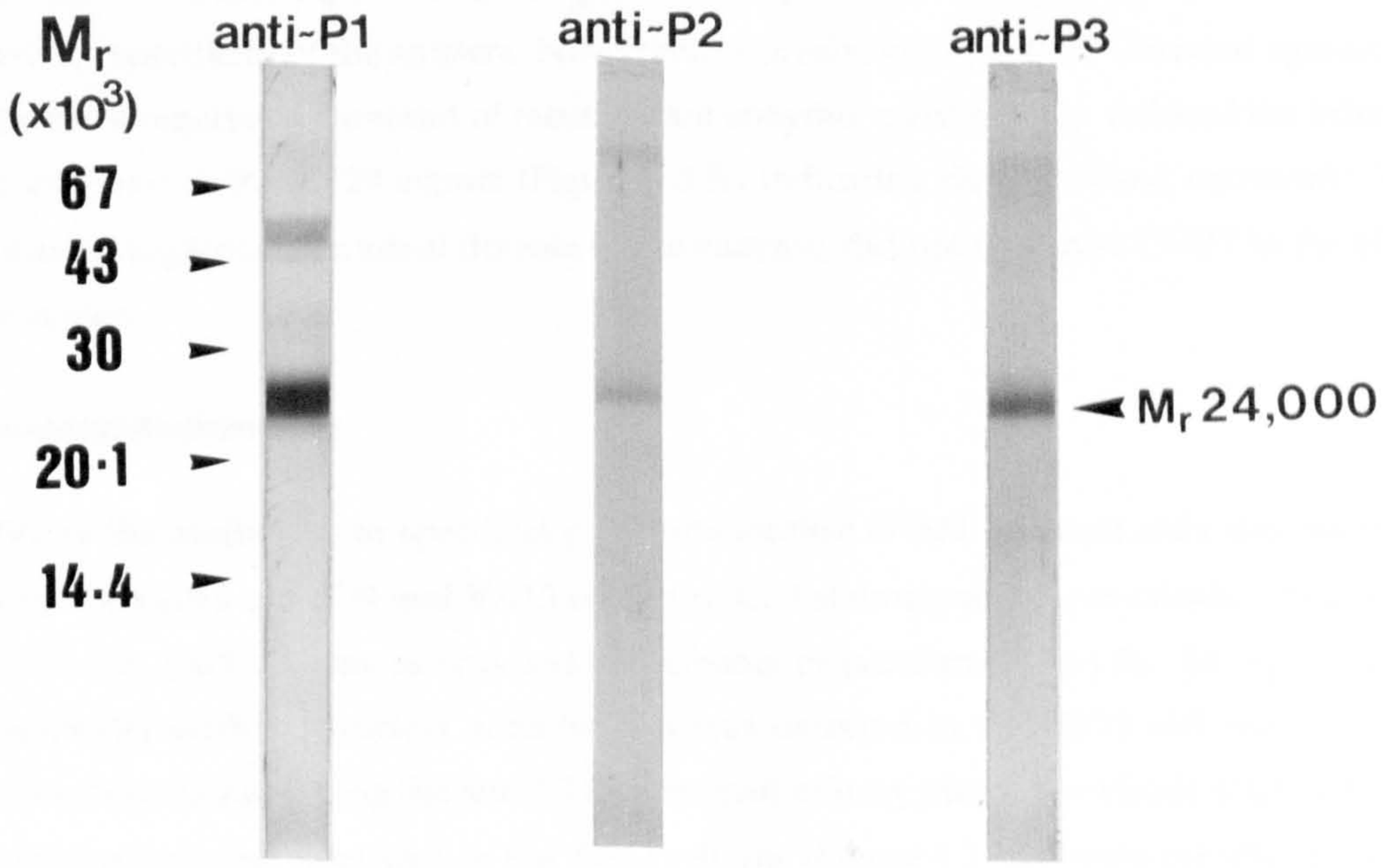


Figure 4.3.3d: Methylated rO^6-MT detected on electroblot strips by the 3 different anti-peptide antibodies. Anti-P1 (directed against the N-terminus of the enzyme), anti-P2 (directed against an internal site) and anti-P3 (directed against the C-terminus) antibodies. 8.8ng of rO^6-MT in each lane subjected to SDS-PAGE and electroblot strips were probed with : anti-P1 (3μg/ml), anti-P2 (7μg/ml) anti-P3 (3μg/ml) antibodies (all affinity-purified on peptide columns).

HT29 cells which contain high levels of the enzyme, and VA13 cells which have no detectable enzyme activity. Ant-P1 (N-terminus) and anti-P3 (C-terminus) anti-peptide antibodies detected O^6-MT in HT29 cell extracts as evidenced by a band of M_r 24,000. This band was not present in VA13 extracts (Figure 4.3.3e and f), demonstating that the antisera are able to detect O^6-MT in crude cell extracts. Clearly these antisera cross-react with other proteins in the crude cell extracts as indicated in Figure 4.3.3e where a band of 18,000 is also detected by the N-terminal antibody in both the HT29 and VA 13 cell extracts. However, no M_r 24,000 band, corresponding to O^6-MT was seen with preimmune sera which had been purified by protein A-Sepharose chromatography or by immunopurification through a relevant peptide affinity column. Insufficient rO^6-MT was available for further preabsorption experiments using excess enzyme to block antibody reaction in order to further test the specificity of the antisera. Nevertheless preabsorbing anti-P3 (directed against the C-terminal) with an equivalent amount of recombinant enzyme convincingly reduced the intensity of the M_r 24,000 band in the HT29 extract (Figure 4.3.3f) indicating that this band represents O^6-MT. Anti-P2, directed against an internal domain of the enzyme, did not recognise O^6-MT in the HT29 or VA13 cell extract.

4.3.4 Immunocytochemistry

The ability of the antibodies to specifically immunolocalise O^6-MT in intact cells was assessed by immunocytochemistry of HT29 and VA13 cell lines. Cell staining was most intense when the cells had been fixed in 100% acetone as opposed to methanol or paraformaldehyde. Strong cytoplasmic immunoreactivity with perinuclear accentuation was detected in the HT29 cell line but with no associated nuclear staining using the anti-P3 (C-terminal) affinity purified antibody (Figure 4.3.4a). In contrast minimal staining was seen in the VA13 cell line (Figure 4.3.4b). Some cytoplasmic staining was seen in HT29 cells with preimmune serum, but only following concentration of the serum on a protein-A Sepharose column. Preimmune sera which had been 'sham' affinity-purified on a column of immobilised peptide did not stain HT29 cells (Figure 4.3.4c). No staining was seen in either cell line with anti-P1 or anti-P2 affinity purified antibodies.

4.3.5 Immunohistochemistry

None of the three antibodies developed were able to detect O^6-MT in frozen liver tissue or paraffin-embedded liver tissue sections despite the use of different tissue fixatives and protease digestion of the tissue sections.

4.4 Discussion

Antibodies to O^6-MT were first developed in 1990 when monoclonal antibodies were raised against partially-purified human O^6-MT (Brent et al, 1990). Subsequent cloning of the full length O^6-MT coding DNA has now allowed other groups to develop anti-peptide antibodies to regions of the predicted protein sequence (Pegg et al, 1991a; Ostrowski et al, 1991b). These antibodies have been characterised in Western blotting but have not been used to immunolocalise the protein in tissue

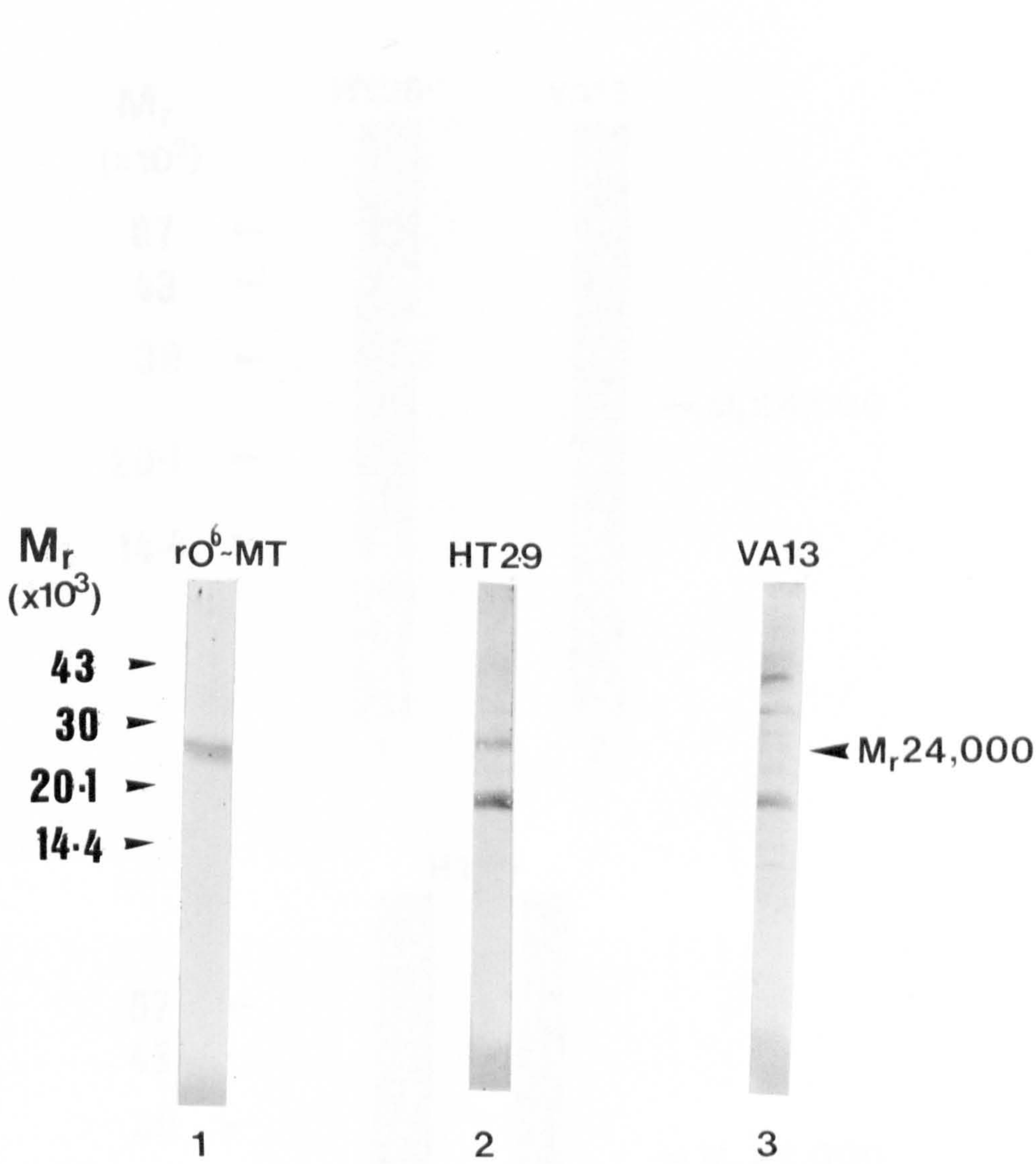


Figure 4.3.3e: SDS-PAGE/immunoblotting of crude extracts prepared from an enzyme proficient and enzyme deficient cell line. 80µg of extract protein from HT29 (O^6-MT proficient) and VA13 (O^6-MT deficient) cells, and rO^6-MT. Electroblot strips were probed with anti-P1 (N-terminal directed) antibody used at a concentration of 3µg/ml. Lanes 1-3 contain 100fmol, 112fmol, and 0fmol of O^6-MT respectively predetermined by direct enzyme assay .

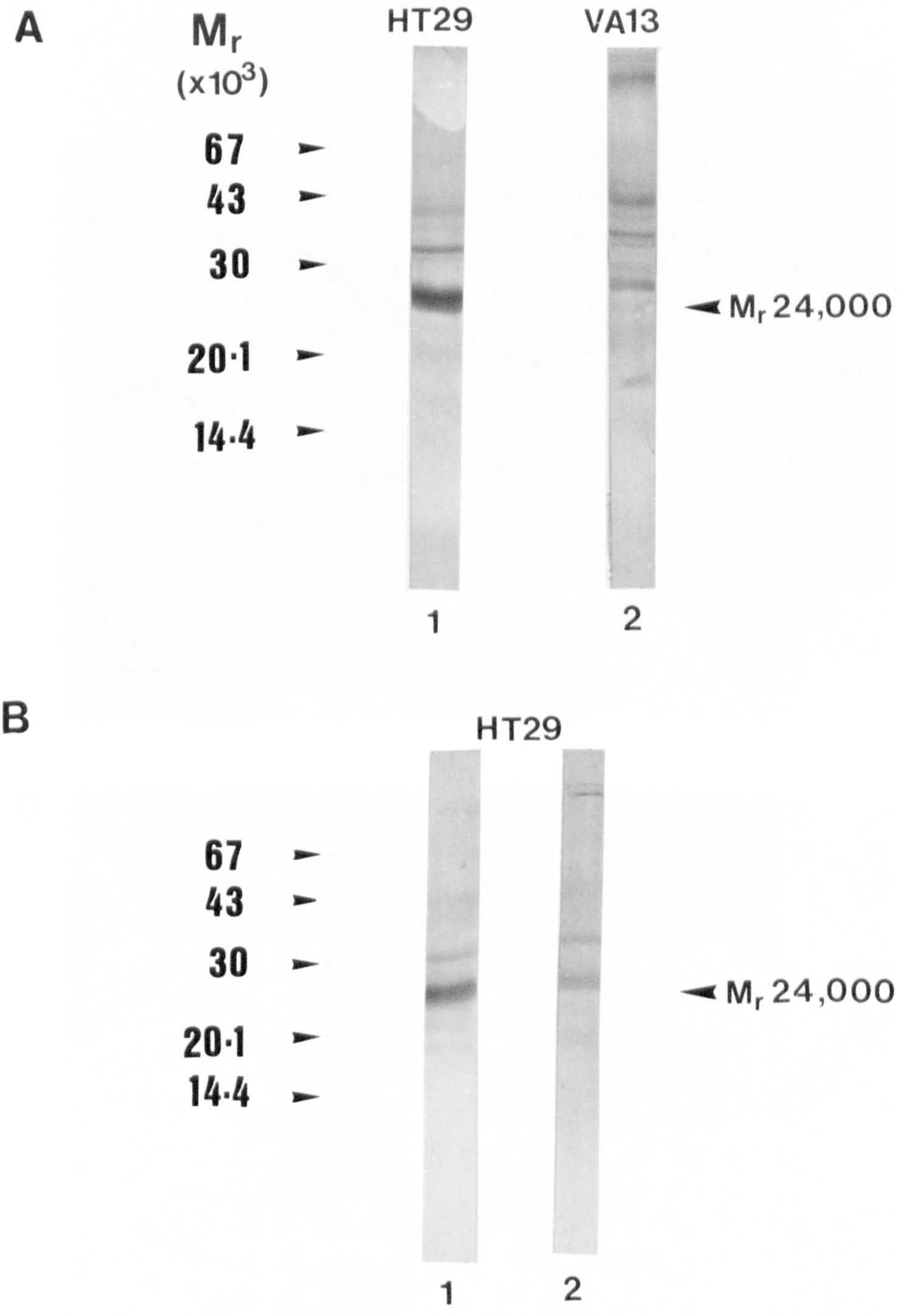


Figure 4.3.3f: SDS-PAGE/immunoblotting of crude extracts prepared from an enzyme proficient and enzyme deficient cell line. 80µg of extract protein from HT29 (O^6-MT proficient) and VA13 (O^6-MT deficient) cells. Electroblot strips were probed with anti-P3 (C-terminal directed) antibody at a concentration of 3µg/ml. (A) Lane 1: HT29 (112fmol of O^6-MT determined by direct enzyme assay); Lane 2: VA13 (no O^6-MT detected by direct enzyme assay). (B) HT29 extract (112fmol) run in both lanes and probed with anti-P3 antibody (3µg/ml; lane 1); and anti-P3 (3µg/ml) antibody preabsorbed with an equal molar amount of rO^6-MT protein (lane 2).

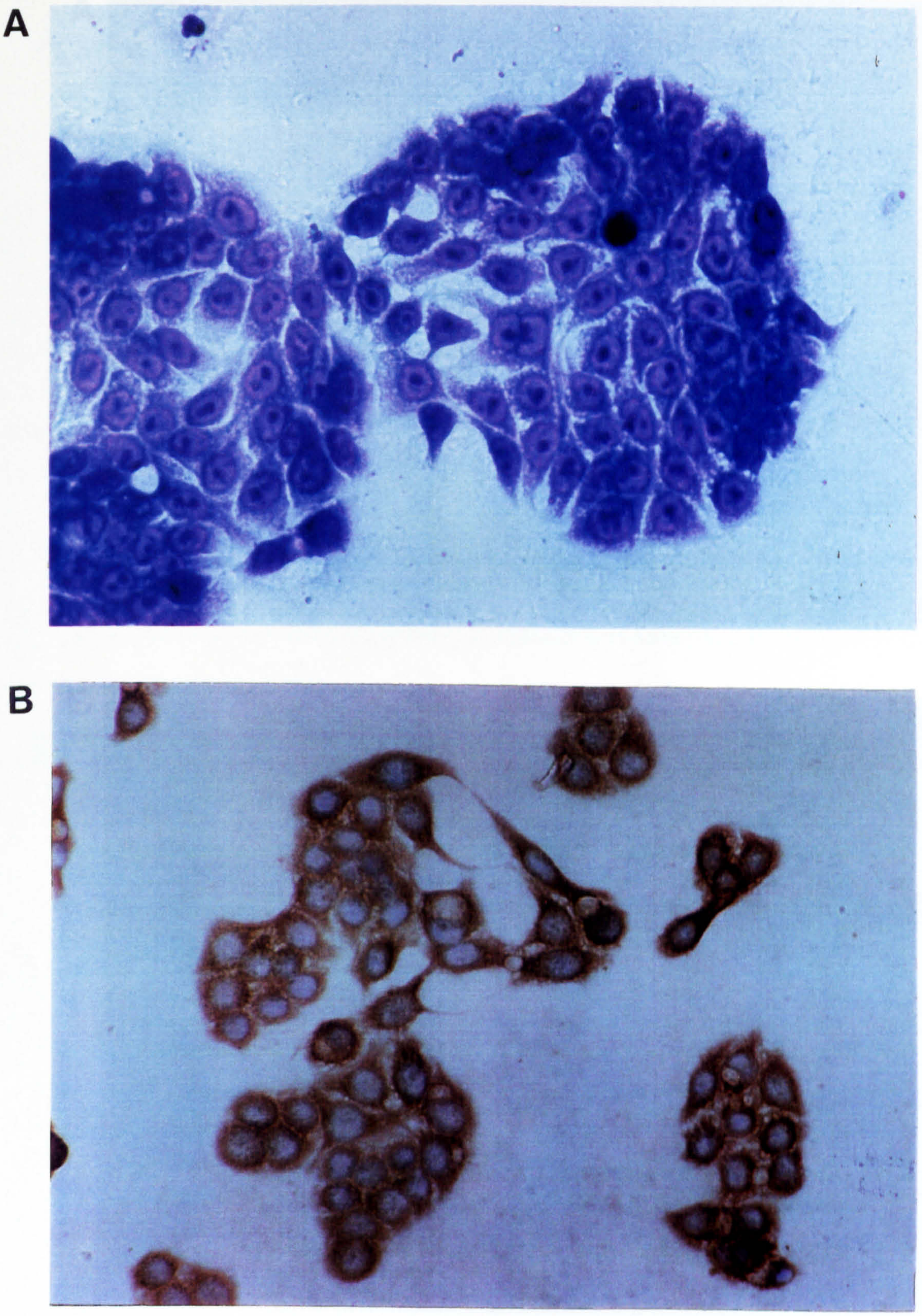


Figure 4.3.4a: HT29 cell line, containing 1330fmol/mg protein of O^6-MT as determined by enzyme assay. (A) Stained with haematoxylin and eosin to show cell morphology. (B) Strong cytoplasmic immunoreactivity in HT29 cells with immunopurified anti-P3 antibody (directed against the C-terminus of O^6-MT). Anti-P3 used at a concentration of 30µg/ml.

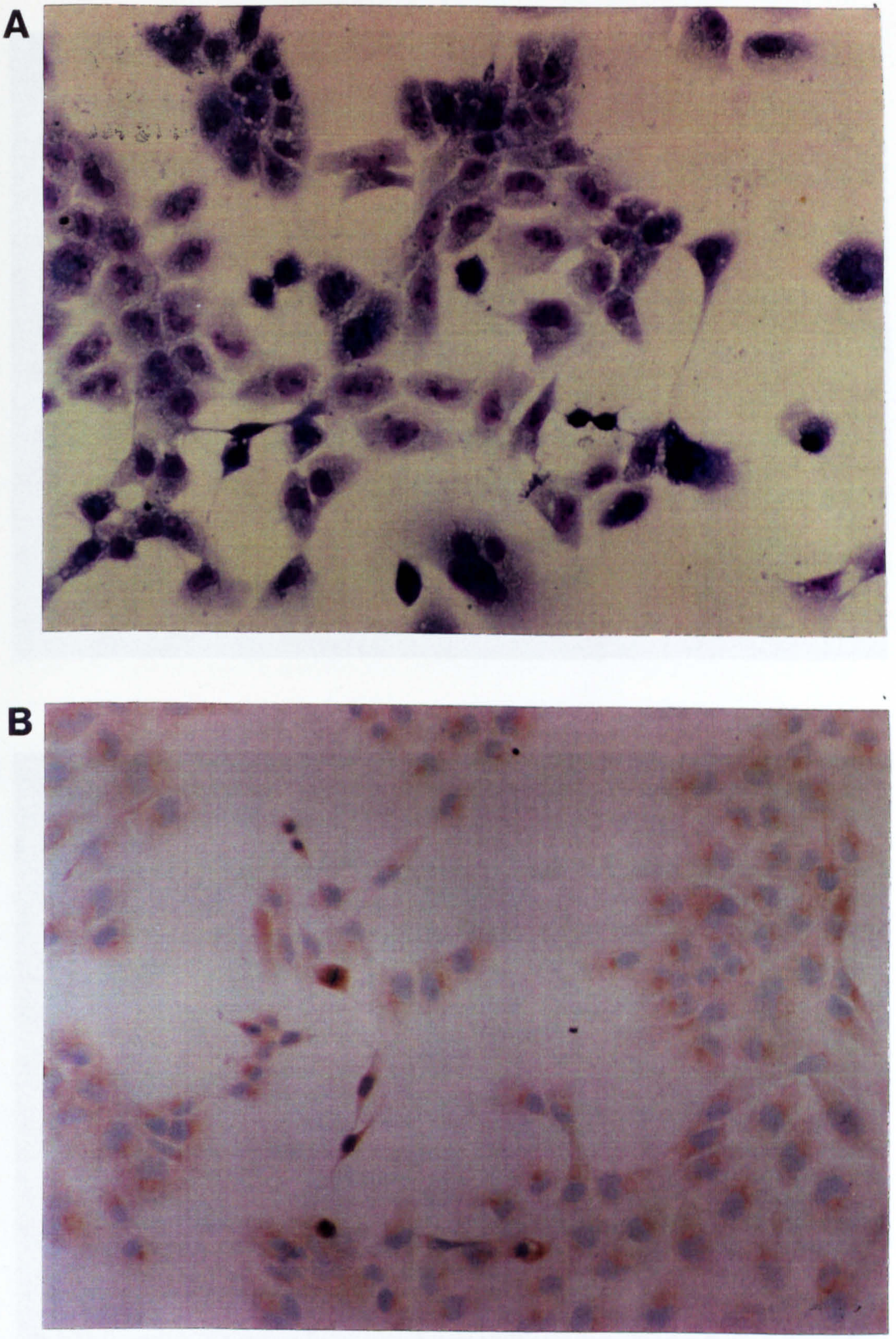
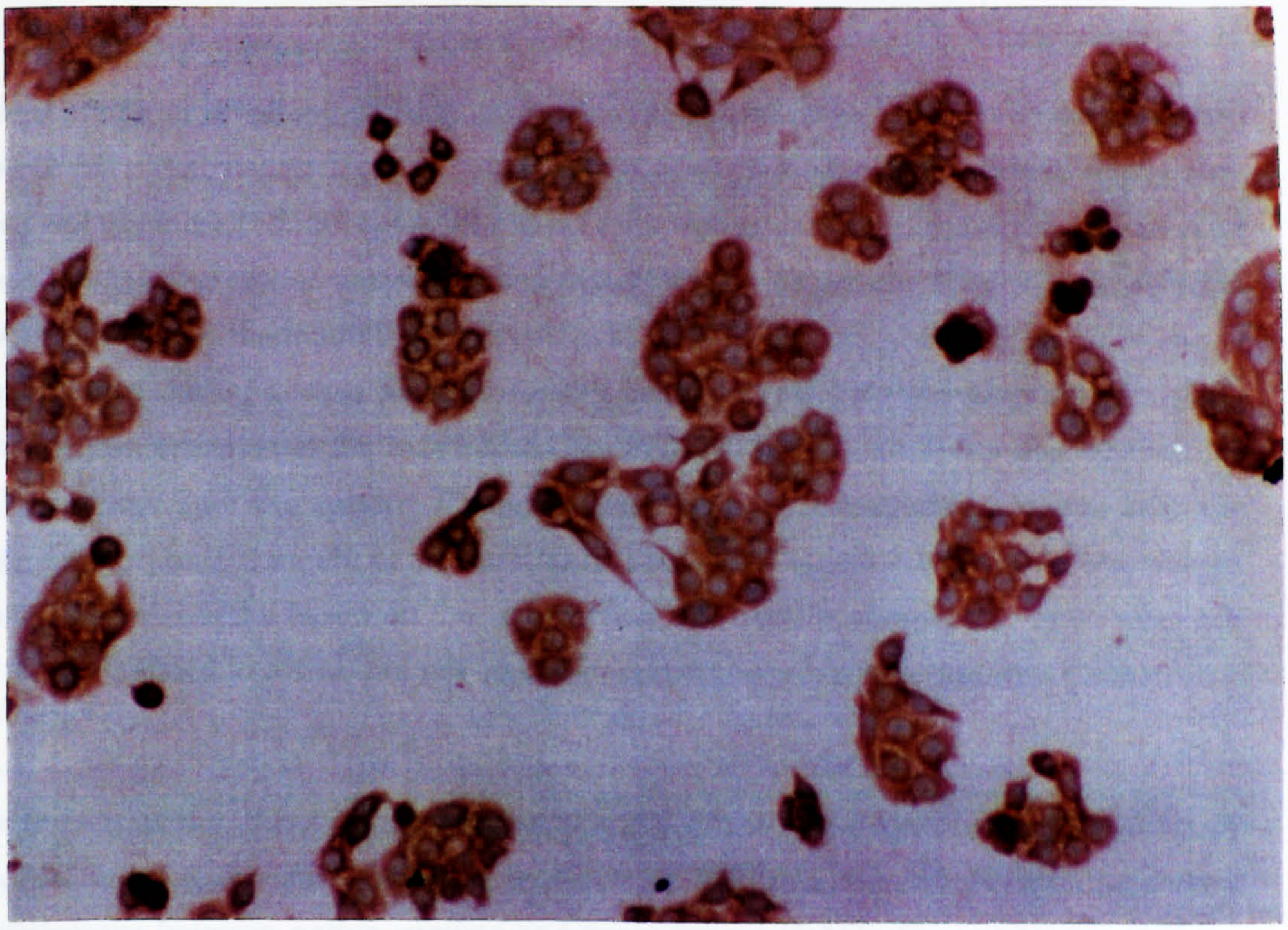


Figure 4.3.4b: VA13 cell line, containing undetectable levels of O^6-MT as determined by enzyme assay. (A) Haematoxylin and eosin staining of the cell line to show cell morphology. (B) Minimal cytoplasmic immunoreactivity with anti-P3 antisera (directed against the C-terminus of O^6-MT). Anti-P3 used at a concentration of 30µg/ml.

A



B

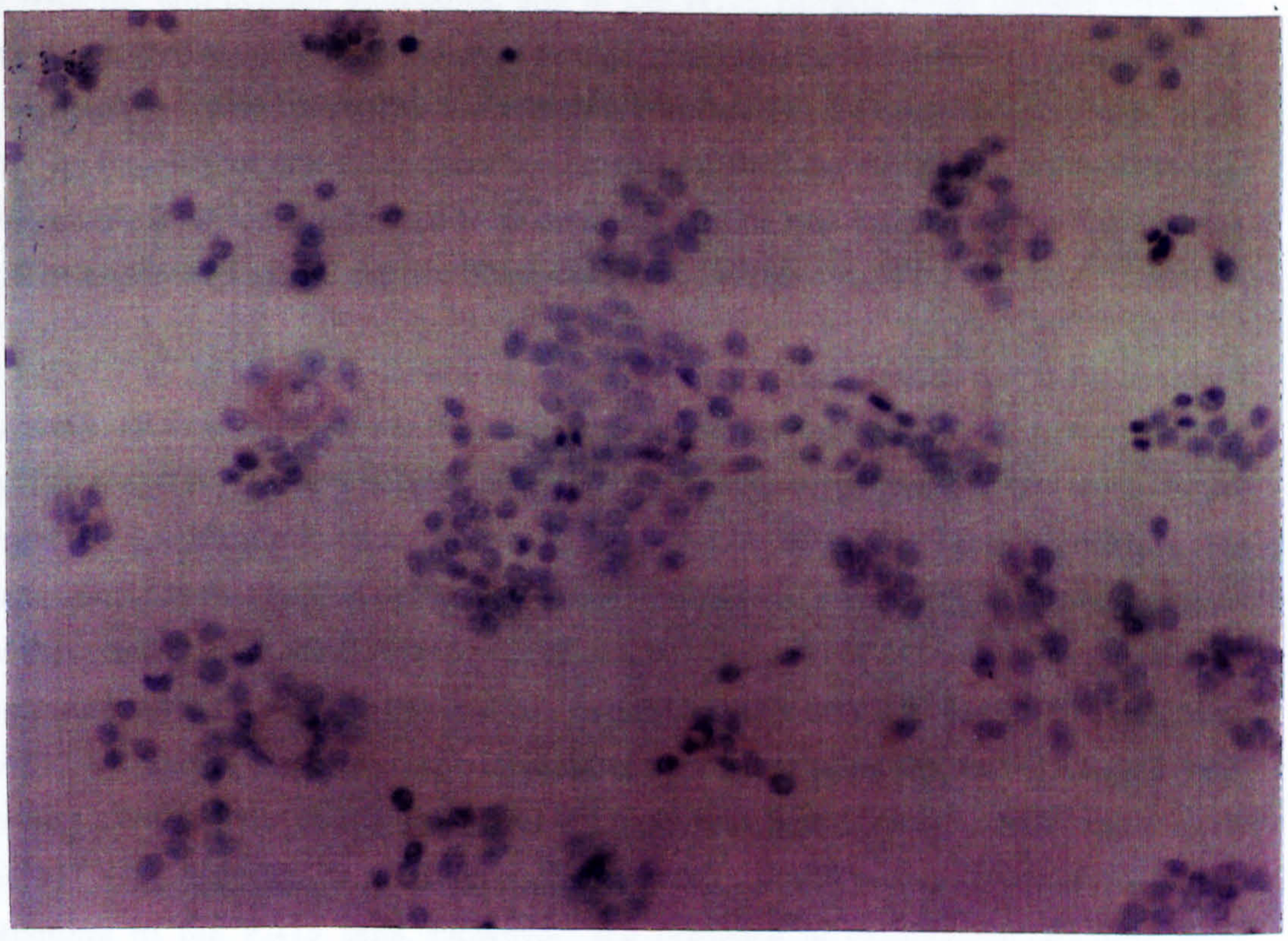


Figure 4.3.4c: Specificity of cell staining with anti-P3 antibody. (A) Cytoplasmic staining in cells from the HT29 cell line with anti-P3 antisera (directed against the C-terminus of O^6-MT). (B) Absence of immunoreactivity in the same cells with preimmune sera.

sections.

In the present study all three anti-peptide antibodies recognised native (non-methylated/active) recombinant O^6-MT. Methylation of recombinant protein, as a result of enzyme action on methylated radiolabelled calf thymus DNA, did not substantially affect the tertiary structure of the protein as all three antibodies were also able to detect the methylated form of the protein. However, the reaction against the three sites on the methylated enzyme was less intense suggesting that either loss of enzyme had occured during sample concentration by methanol/chloroform precipitation or some minor change in conformation of the enzyme had occured following methylation that affected the affinity of the antibody for the epitope. Conformational change on methylation occurs with the bacterial *ada* gene product (Lindahl et al, 1988), and it has been suggested that a similar change occurs in the human enzyme based on the observation that certain anti-peptide antibodies can precipitate the methylated (inactive) but not native (non-methylated/active) enzyme (Ostrowski et al, 1991b).

That two of the antibodies, those recognising the N and C-terminal domains but not the antibody recognising the internal domain, were able to detect O^6-MT in crude cell extracts suggests a structural difference between native enzyme and recombinant O^6-MT. The specificity of these antibodies was confirmed by the absence of detectable enzyme at M_r 24,000 in extracts from the VA13 cell probed with the anti-peptide antibodies, and in the HT29 extracts when reacted with pre-immune sera. However the antibodies also cross reacted with other protein bands on immunoblots, as has been reported by other groups. This limits their usefulness for analysis of the enzyme in crude cell extracts. The inability of anti-P2 antisera to recognise O^6-MT in the HT29 cell extract was unexpected as a polyclonal antibody raised to an identical peptide was shown to detect the enzyme in a crude cell extract from the CEM cell line (Ostrowski et al, 1991b).

The intense cytoplasmic/perinuclear staining of HT29 cells by antibody recognising the C-terminus of O^6-MT is of interest as in the course of this work cytoplasmic staining of human hepatocytes was observed by Lee et al (1992) using a polyclonal antibody raised against recombinant protein. As the antibody binds non-specifically to other proteins in crude HT29 cell extracts on immunoblots, it is not possible to conclude that such staining represents evidence of O^6-MT within the endoplasmic reticulum of these cells. Nevertheless, the specificity of the staining for O^6-MT was supported by the negligible immunoreactivity seen in the enzyme deficient VA13 cells. It should be noted that although VA13 cell enzyme activity is undetectable using the enzyme assay reported in Chapter 3 the cells do contain a small amount of enzyme, about 400 molecules /cell (Wu et al, 1987) and a small amount of O^6-MT RNA, detected by polymerase chain reaction, which would account for some of the weak staining in the VA13 cells. However, the presence of cytoplasmic staining with concentrated pre-immune IgG suggests that some of this labelling is non-specific and this is supported by the lack of staining with 'sham' peptide affinity-purified pre-immune sera which removed non-specific binding immunoglobulin.

Although the expected location of O^6-MT immunoreactivity is nuclear, as O^6-MT repairs DNA, the presence of cytoplasmic staining does not exclude specific O^6-MT detection as the intracellular distribution of the enzyme in human tissues is still unknown. Subcellular distribution of O^6-MT in rat liver has been addressed using enzyme assays of subcellular fractions. The cytosol has variously been reported to contain 35% (Jun et al, 1975), 59% (Pegg et al, 1983) and 72% (Hora et al, 1983) of total cellular activity. O^6-MT has also been isolated from rat liver mitochondria (Myers et al, 1988). More recently Ayi et al (1992) have reported immunolocalisation of O^6-MT in human cell lines using a polyclonal antibody raised against recombinant protein. Although staining was predominantly nuclear some cell lines showed cytoplasmic immunoreactivity. Whether or not any O^6-MT is ever cytoplasmic in location *in vivo* remains unclear. The reported distribution of O^6-MT may just represent leakage of nuclear enzyme into the cytoplasm during tissue preparation and fractionation. It is possible, however, that there are different forms of the enzyme in different cellular compartments and it has been suggested that the cytoplasmic enzyme may be a mutant form of the nuclear enzyme that lacks a nuclear localisation sequence and is, thus, non-functional because of its inability to access nuclear DNA (Ayi et al, 1992). This is an important consideration as the sensitivity of such cells to alkylating agents might not be reflected by quantification of levels by direct enzyme assay or other approaches that involve measurement of total cellular O^6-MT activity.

The inability to detect O^6-MT in liver sections immunohistochemically reflects the difficulty in producing antibodies that detect epitopes under a variety of conditions, such as O^6-MT bound to a solid phase in the ELISA approach, denatured in SDS-PAGE and altered by tissue fixatives in paraffin-embedded tissue sections. Although in the present study epitopes on O^6-MT might have been masked during tissue fixation such an explanation does not account for the inability to detect the enzyme in frozen liver tissue. It is, however, not uncommon for anti-peptide antibodies to detect denatured protein but not native antigen *in vivo*. Using anti-peptide antibodies of similar specificity other groups have also failed to detect the enzyme in tissue sections (Pegg et al, 1991a; Ostrowski et al, 1991b). While the present study was in progress, Lee et al (1992) managed to detect O^6-MT in paraffin-embedded sections of normal human liver using antibodies raised against recombinant enzyme; they did not study any form of liver disease. The enzyme was localised to the nucleus in most cells but in some cells there was coexistant cytoplasmic staining, as has also been reported occur in human cell lines (Ayi et al, 1992). Although staining was confined to the hepatocytes, with little immunoreactivity in non-parenchymal cells, not all hepatocytes expressed O^6-MT. These findings indicate the importance of comparing levels of O^6-MT from whole tissues extracts with the cellular distribution of the enzyme. Normal liver, a tissue with one of the highest levels of O^6-MT activity, would be expected to be relatively resistant to the effects of alkylating agent damage but the presence of hepatocytes which do not contain immunoreactive enzyme indicates that there is a subgroup of cells which are likely to be more susceptible to DNA base damage than is suggested by the enzyme assay findings.

Two of the antibodies produced in this study detected the native (non-methylated/active) and

methylated (inactive) O^6-MT in immunoblots of enzyme -containing cell extracts. These antibodies, directed against two distinct parts of the enzyme, namely the N- and C-termini, should therefore be useful in future studies characterising O^6-MT in cirrhotic liver. These antibodies provide a means of establishing a quantitative method for measuring O^6-MT by SDS-PAGE/immunoblotting. This could potentially enable the total amount of enzyme *in vivo* (native and endogenously methylated) to be measured immunochemically in extracts. An estimate of the amount of methylated (inactive) enzyme present in the extract could then be obtained by subtracting the amount of native (non-methylated/active) enzyme, calculated from the direct enzyme assay, from this value. The level of methylated enzyme in an extract would be an indirect measure of the tissue's exposure to endogenous alkylating agents; that is a "fingerprint" of the extent of recent DNA damage; this is considered further in Chapter 5.

4.5 Summary

Production and characterisation of anti-peptide antibodies to O^6-MT

- Polyclonal anti-peptide antibodies raised to regions of the enzyme predicted to be antigenic:
    - N-terminal (amino acids 1-20; anti-P1)
    - Internal site (amino acids 171-183; anti -P2)
    - C-terminal (amino acids 193-207; anti-P3)
- SDS-PAGE and Immunoblotting:
    - All antibodies specifically detected native (non-methylated) and methylated (inactive) human recombinant O^6-MT
    - N-terminal and C-terminal directed antibodies detected O^6-MT in crude cell extracts of enzyme-rich HT29 cells but not in extracts of enzyme-deficient VA-13 cells confirming antibody specificity.
- Immunocytochemistry:
    - Cytoplasmic staining of HT29 cells with C-terminal antibody
- Immunohistochemistry
    - None of the antibodies detected O^6-MT in frozen or paraffin-embedded liver tissue sections and therefore cannot comment on the heterogeneity of enzyme expression in the liver.

Chapter 5

Characterisation Of O^6-methylguanine-DNA methyltransferase In Cirrhosis

5.1 Introduction

Elucidation of the physicochemical properties of O^6-methylguanine-DNA methyltransferase (O^6-MT) have been hindered by its relatively low tissue abundance and the notorious instability it displays when subjected to protein fractionation. The enzyme has therefore only recently been fully purified and has not as yet been subjected to physicochemical analysis (Brent et al, 1990). Nevertheless, the O^6-MT gene has been cloned and the predicted amino acid sequence determined which is comparable with sequences obtained from partial protein purification (Tano et al, 1990; von-Wronski et al, 1991). Physicochemical studies of the recombinant enzyme have been undertaken (Koike et al, 1990) and it is likely that knowledge of the tertiary structure of O^6-MT will come from further analysis of the recombinant enzyme.

Although levels of the enzyme vary widely between human tissues, it is not known whether these differences are reflected in structural differences in the enzyme, the presence of more than one form of the enzyme, as is the case in procaryotes, or different rates of enzyme turnover in different tissues. Larger molecular weight forms of the enzyme have been reported in human lymphoid tissue (Major et al, 1990) and rat liver (Myers et al, 1988) but the significance of these larger forms in terms of the setting of tissue enzyme levels is unclear. Native O^6-MT acts by removing methyl groups from the O^6 position of guanine in DNA which it transfers to its own specific cysteine residue and thus in the process becomes both methylated and inactivated. The fate of the methylated enzyme, which is not regenerated, is not known, although a recent study has suggested that it is rapidly degraded (Pegg, 1991b).

Although human liver has one of the highest tissue levels of O^6-MT little is known about the structure of the enzyme in normal liver. It is not clear whether structural changes occur in disease to account for the findings of low levels of native (non-methylated/active) enzyme in human cirrhotic tissue compared to non-cirrhotic diseased liver and normal liver (see Chapter 3). Thus, it is possible that cirrhotic and non-cirrhotic liver contain different enzyme forms which have different activities. C-terminal degradation of O^6-MT has been reported in normal liver (Pegg et al, 1991a) raising the possibility that degradation may be marked in cirrhotic liver leading to the observed lower levels of active enzyme. The availability of extracts from cirrhotic, non-cirrhotic and normal liver has enabled some of these questions to be addressed by SDS-PAGE analysis of the enzyme in these extracts using the techniques of fluorography and immunoblotting with anti-peptide antibodies recognising O^6-MT (whose development was outlined in Chapter 4). These experimental approaches allow the detection of both native O^6-MT and total (native and endogenously methylated/inactive) enzyme in tissue extracts (Figure 5.1). SDS-PAGE/fluorography of extracts, containing [^{3}H-Me]O^6-MT (native enzyme methylated and radiolabelled with tritium), allows native enzyme to be visualised as a band on fluorograms. In contrast SDS-PAGE followed by immunoblotting allows the detection of both native and methylated O^6-MT.

The aim of this part of the study was four -fold: (i) to confirm the direct enzyme assay findings of

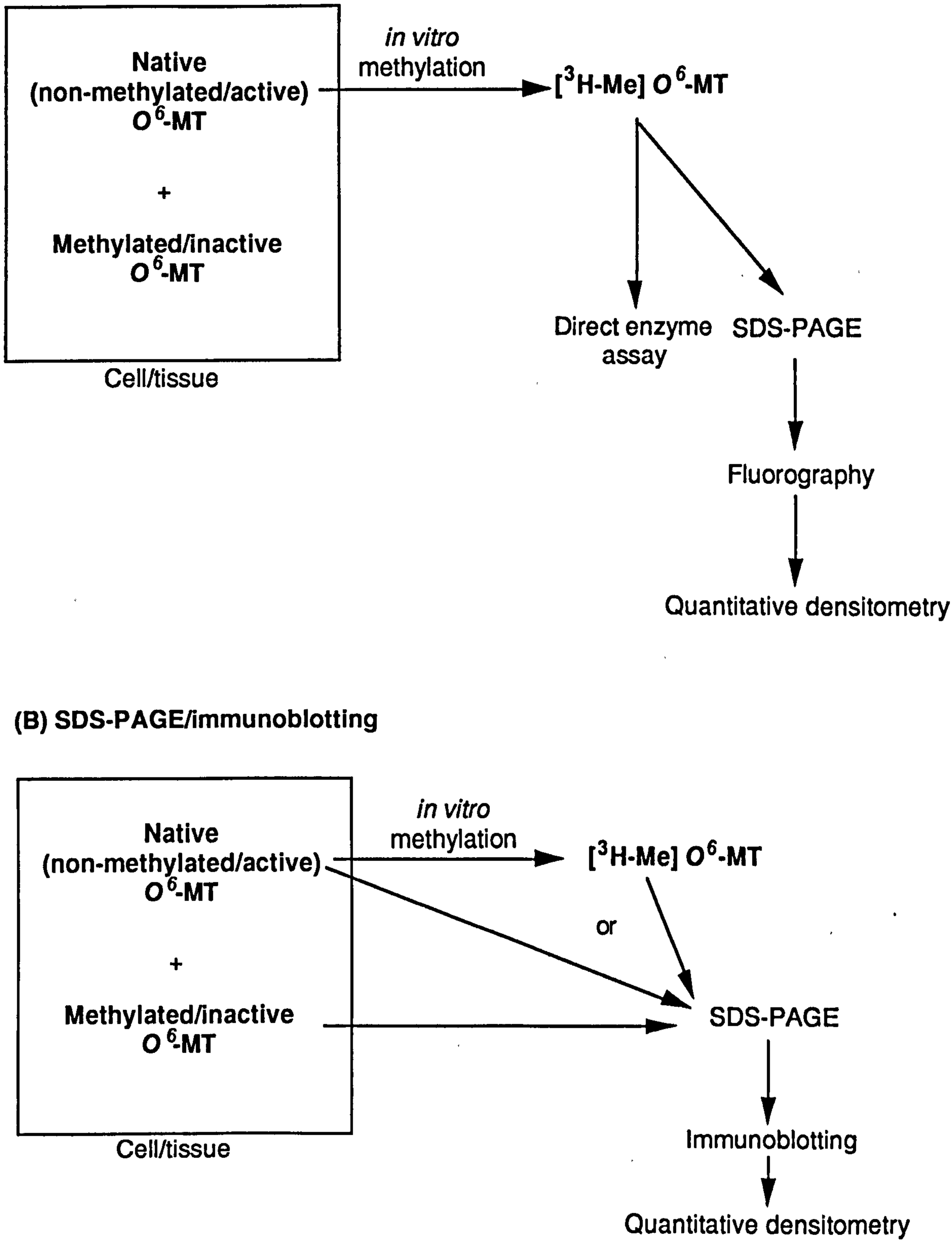


Figure 5.1: Experimental approaches used to detect O^6-MT in tissue extracts.
(A) SDS-PAGE/electroblotting followed by fluorography detecting native enzyme (methylated and radiolabelled in vitro). (b) SDS-PAGE/electroblotting followed by immunochemical detection using antibodies which detect both methylated and non-methylated enzyme.

low levels of O^6-MT in cirrhosis using SDS-PAGE/fluorography and quantitative densitometry; (ii) to establish the presence or absence of different molecular weight forms of the enzyme; (iii) to look for possibility of C-terminal protease degradation of the enzyme; (iv) to compare total enzyme levels (native/non-methylated and endogenously methylated/inactive enzyme) with levels of native enzyme measured by the enzyme assay.

5.2 Methods

5.2.1 Preparation of samples

Liver tissue was available from 36 of the 48 patients studied in Chapter 3 (cirrhotic, n= 15; non-cirrhotic diseased liver, n=17; normal liver, n=4). Lymphocytes from 7 of these patients were also available for study. Liver and lymphocyte extracts were prepared as described previously (Chapter 3). Extracts, where indicated, were incubated with excess tritiated methylated calf thymus DNA (substrate) at 37°C for 90 minutes to methylate and radiolabel all the enzyme in the extract. A repeat direct enzyme assay was carried out on all the extracts (as described in Chapter 3) prior to SDS-PAGE. Extracts were mixed with sample buffer containing Pyronin Y and then subjected to SDS-PAGE (see Chapter 4).

5.2.2 Immunoblotting

Liver and lymphocyte extracts were separated by SDS-PAGE in 1.5mm non-gradient 15%polyacrylamide gels under reducing conditions, blotted on to 0.2µm nitrocellulose membrane that was subsequently probed with N- and C-terminal directed anti-peptide antibodies as described in Chapter 4.

5.2.3 Fluorography

Human recombinant O^6-MT and cell and tissue extracts produced to contain [^{3}H-Me] enzyme were subjected to SDS-PAGE using 15% polyacrylamide gels as described in Chapter 4. [^{14}C] labelled low molecular weight protein markers (GIBCO BRL) were run on all gels. Following electrophoresis, gels were electroblotted onto polyvinylidene difluoride (PVDF)(BioRad) rather than nitrocellulose membrane. PVDF was used according to the manufacturers instructions and following electroblotting was washed in water, allowed to dry and then sprayed with 'En^{3}hance' spray (New England Nuclear-Dupont) and redried. X-ray film (Fuji RX) was preflashed from a distance of 45cm using a 'Sensitize' preflashing gun (Amersham) in order to increase the sensitivity of the film to low light emissions (Roberts, 1985). The PDVF membrane was then exposed to the x-ray film at -80°C for 4 weeks. The linearity of the response of the film to low intensity emissions following preflashing was established in preliminary work (personal communication; Dr G N Major).

5.2.4 Densitometry

Densitometric analysis of the fluorograms and immunoblots were performed on a BioImage laser

densitometer (Millipore, USA) linked to a Sun computer. The fluorograms were analysed using "whole band analysis" and the immunoblot strips by "1-D analysis". Data are expressed as an integrated optical density (IOD) which is a measure of the overall band intensity. The molecular weight of the bands on fluorograms and immunoblots were determined by measuring their Rf value which was compared with those of the protein standards run on each gel.

Statistics were performed as described in Chapter 3.

5.3 Results

5.3.1 Levels of O^6-MT in cirrhosis

SDS-PAGE/fluorography/densitometry provides not only another method for measuring the level of native O^6-MT in tissue extracts but it has the advantage of also allowing physicochemical characterisation of the enzyme. A band of M_r 24,000, representing [^{3}H-Me] O^6-MT was seen on all fluorograms. This indirect method of measuring the native enzyme was calibrated by showing that a linear relationship existed between integrated optical density , a measure of the intensity of the M_r 24,000 band, and the amount of O^6-MT in the HT29 extract (Figure 5.3.1a). The amount of O^6-MT detected by SDS-PAGE/fluorography/densitometry varied by 2-4% on different days, comparable with the direct enzyme inter-assay variation of 2-4% previously reported by Major et al (1990).

Enzyme levels in the liver extracts, as determined by the direct enzyme assay (see Chapter 3), correlated with the Mr 24,000 band intensity (IOD) associated with the fluorograms (r=0.78; p=0.0001). Native O^6-MT levels differed significantly between cirrhotic, non-cirrhotic and normal liver extracts (ANOVA; F=10.89, p=0.0002) when measured by SDS-PAGE/fluorography/ densitometry. O^6-MT was significantly lower in cirrhotic tissue extracts compared with non-cirrhotic (p=0.0002) and normal liver extracts (p=0.0015) (Figure 5.3.1b). These findings are, therefore, comparable with O^6-MT levels detected using the direct enzyme assay as described in Chapter 3.

5.3.2 Multiple molecular weight forms of O^6-MT

In addition to the expected band of M_r 24,000, representing O^6-MT, lower molecular weight forms were also seen following SDS-PAGE/fluorography. In the liver extracts up to three additional bands were consistently visualised at M_r 23,200, M_r 21,800 and M_r 20,900. All four molecular weight forms were seen in cirrhotic, non-cirrhotic and normal liver (Figure 5.3.2a). Smaller bands of M_r 23,200; 22,500; 21,800; 19,500; 17,900; 16,800; 15,400; 13,400 and 9,400 were also detected in lymphocyte extracts and in recombinant enzyme; and compared with liver, a larger number of bands with different molecular weights were apparent (Figure 5.3.2b). The finding of smaller enzyme forms may be due to either endogenous protease action or different distinct forms of the enzyme. Repeat direct enzyme assay of the lymphocyte extracts prior to SDS-PAGE showed that no loss of activity had occurred during storage, indicating that appearance of the lower molecular weight enzyme forms in

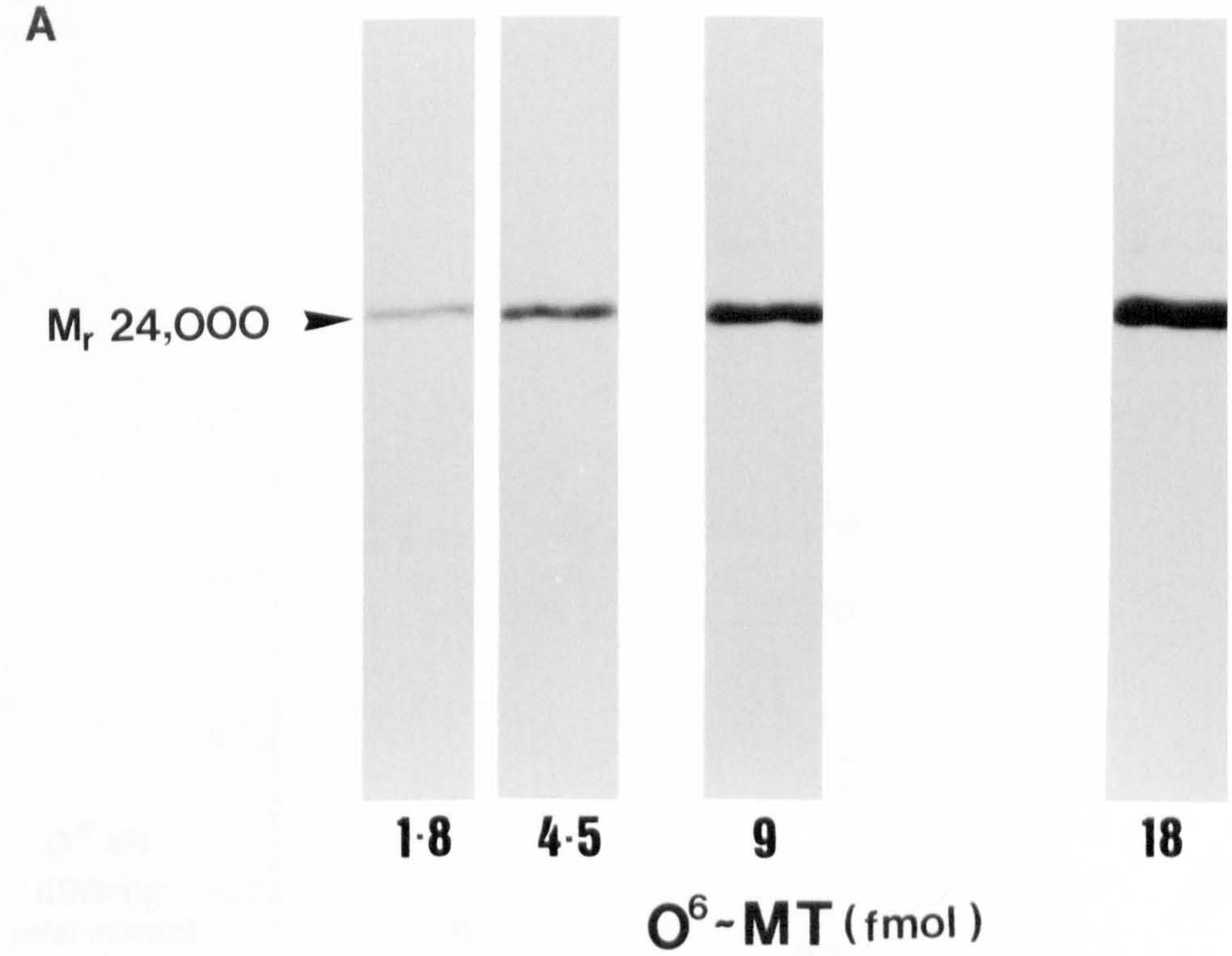


Figure 5.3.1a: Calibration of SDS-PAGE/fluorography/densitometry for quantitating native O^6-MT. (A) Different amounts of HT29 cell extract subjected to SDS-PAGE/electroblotting followed by fluorography (B) Densitometric analysis of M_r 24,000 bands shown in A, expressed as an integrated optical density (IOD). The IOD of the bands are compared to the amount of O^6-MT in the extract as determined by the direct enzyme assay.

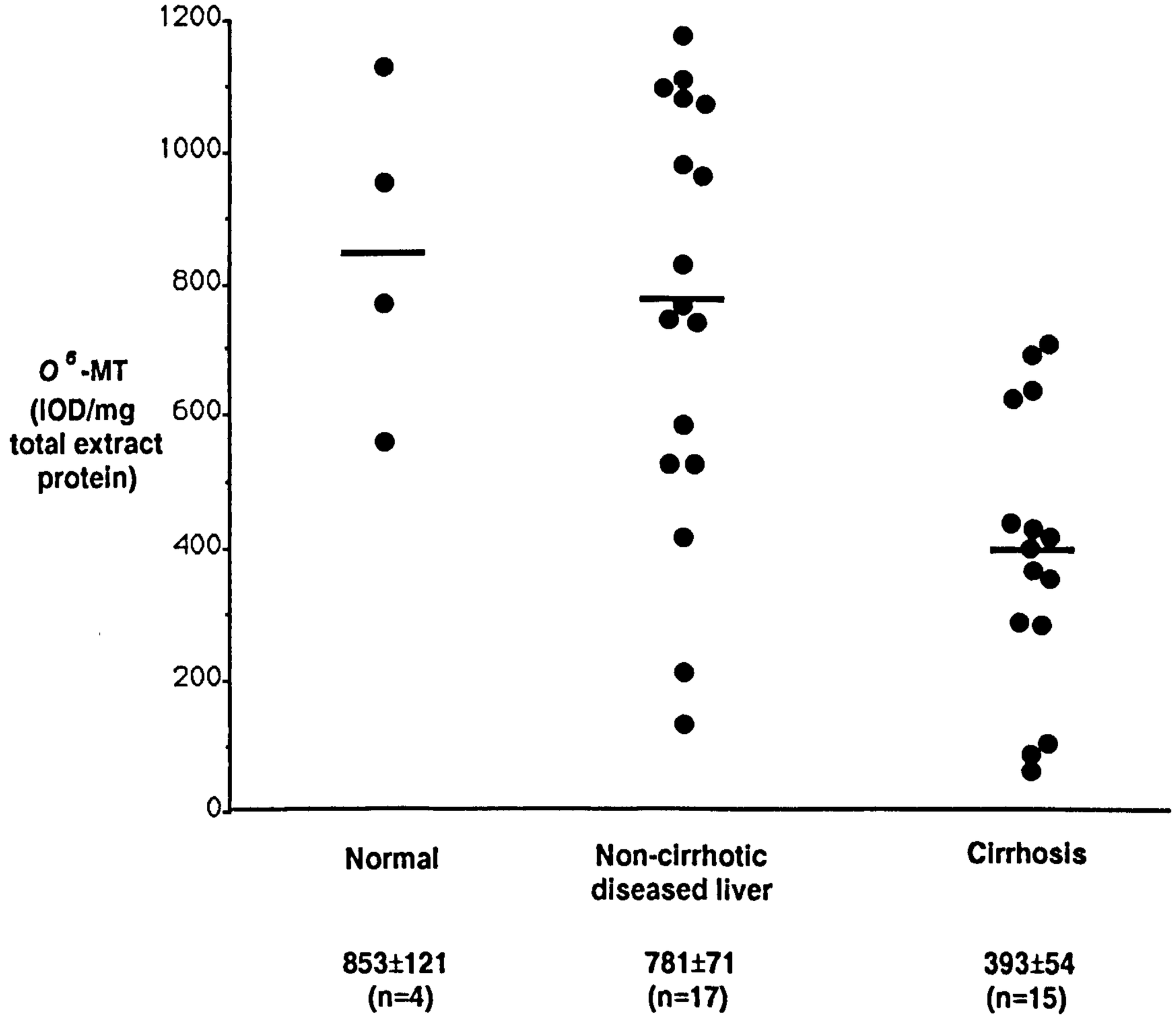


Figure 5.3.1b: Levels of O^6-MT determined by SDS-PAGE/fluorography/densitometry in cirrhotic, non-cirrhotic diseased liver and normal liver. Mean levels are shown by horizontal lines and their values ±SEM are indicated below the graph.

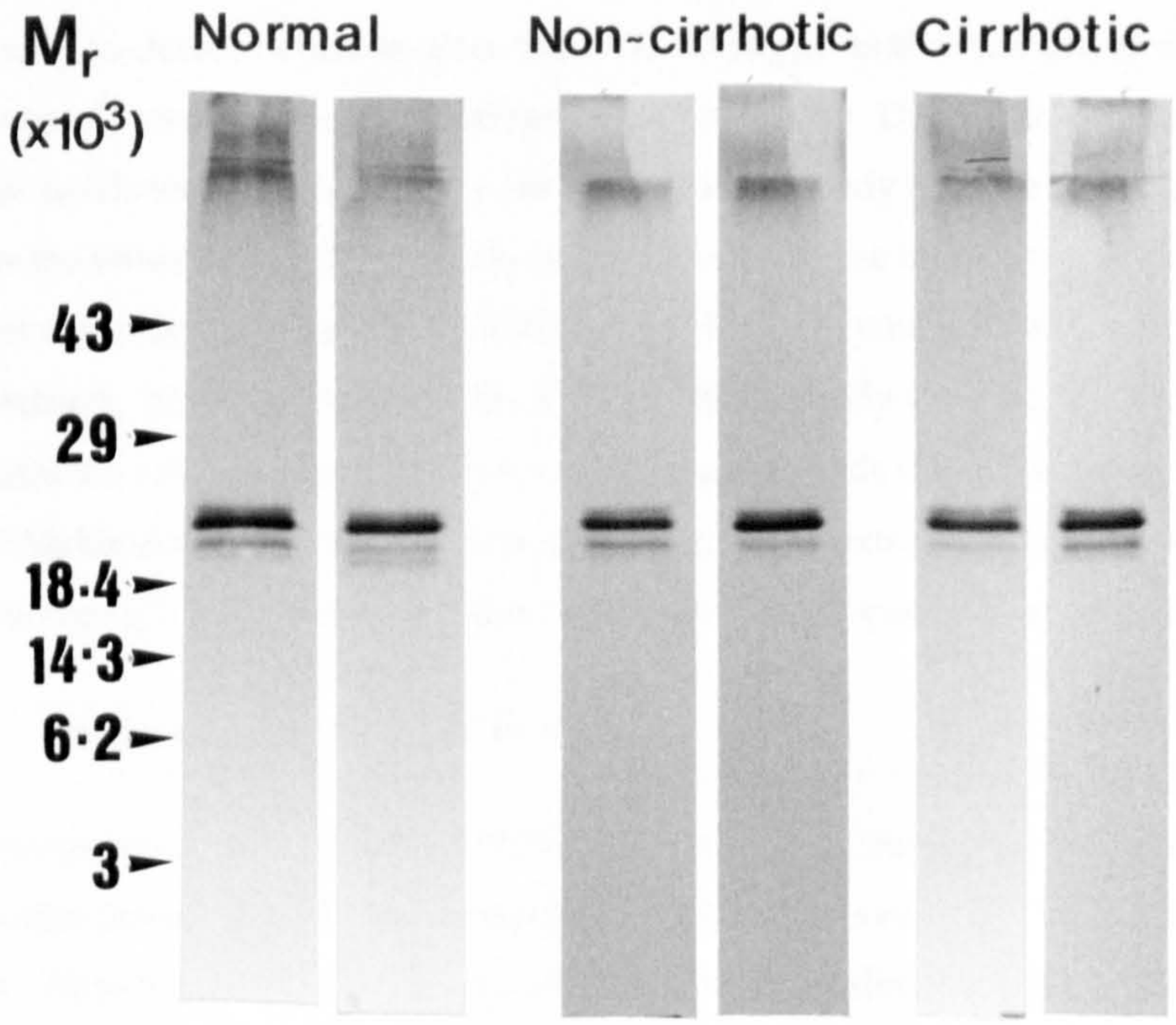


Figure 5.3.2a: Fluorograms of SDS-PAGE/electroblots of liver extracts. Two representative samples from normal, non-cirrhotic and cirrhotic liver are shown.

this enzyme source was not associated with loss of enzyme activity. No molecular weight forms larger than M_r 24,000 were identified by SDS-PAGE/fluorography in either liver or lymphocyte extracts, but a faint band of M_r 27,000 was seen in the HT29 cell extract (Figure 5.3.2b).

5.3.3 C-terminal truncation of O^6-MT

A band of M_r 24,000 was seen with the C-terminal antibody in all liver extracts but not with the N-terminal antibody as the maximum amount of enzyme present was below the level of detection with this antibody. The finding of a single M_r 24,000 band on immunoblots contrasted with the multiple bands of [^{3}H-Me] enzyme detected by fluorography (see above). The inability of the C-terminal antibody to detect bands smaller than 24,000 suggests that the lower molecular weight bands represent C-terminal truncated enzyme (Figure 5.3.3.a). The possibility that these lower molecular weight bands were not detected by the C-terminal antibody because the enzyme was present at levels below the sensitivity of the antibody was ruled out by the findings of a band of M_r 24,000, but not a smaller and more abundant M_r 22,500 band of [^{3}H-Me] enzyme, seen on the corresponding fluorogram of this extract, being detected by the C terminal antibody (lane 7; Figure 5.3.3b). This figure also indicates the value of fluorography in confirming the bands visualised on immunoblots as O^6-MT. The M_r 29,000 band seen on immunoblots of all lymphocyte extracts did not correspond to [^{3}H-Me] O^6-MT (by fluorography) indicating that this represented a non-specific cross reaction(Figure 5.3.3b).

5.3.4 Conformational change in O^6-MT

As previously observed with HT29 cell extract (see Chapter 4) the N-and C-terminal directed antibodies detected O^6-MT on electroblots of SDS-PAGels run with liver extracts at M_r 24,000 (Figure 5.3.2a). Anti-N-terminal antibody seemed unable to detect native enzyme, detecting only the methylated enzyme on electroblots. This suggests that a conformational change may occur in O^6-MT on methylation revealing the N-terminal epitope (Figure 5.3.4). The anti-C-terminal antibody reacted with both forms of the enzyme (native and methylated) in immunoblots. No bands were detected when liver extracts were probed with pre-immune sera, confirming antibody specificity (Figure 5.3.4).

5.3.5 Levels of active and inactive O^6-MT *in vivo*

An attempt was made to establish the immunoblotting procedure as a quantitative and reproducible method for detecting total O^6-MT (native and methylated, normally present *in vivo*). However inter-blot variability in band density when quantitated by densitometry was in the order of 40% probably as a result of variable background staining on the electroblot strips. Nevertheless, while inter-blot variability was excessive, intra-blot reproducibility was ± 2-5 %.

5.4 Discussion

This study using SDS-PAGE/fluorography/densitometry of liver extracts confirmed the previous

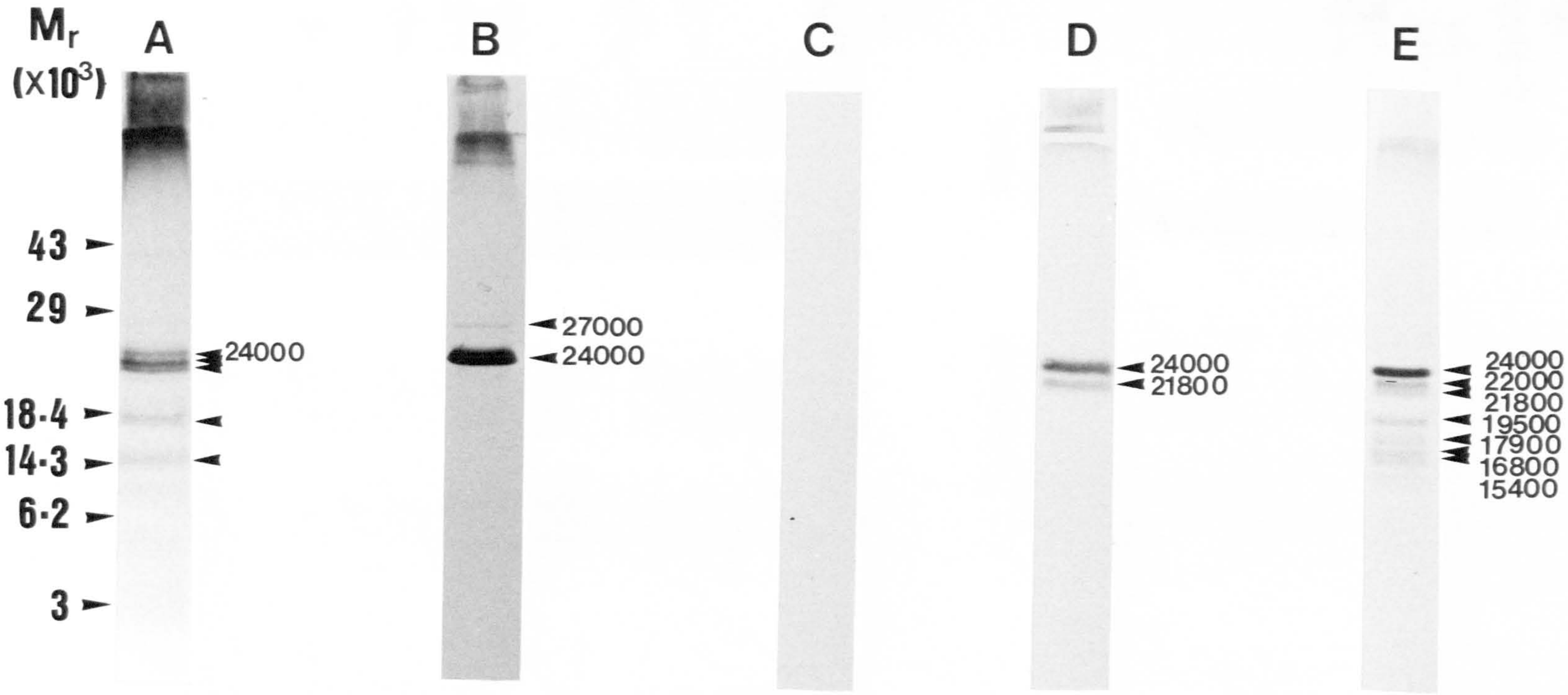


Figure 5.3.2b: Fluorograms of SDS-PAGE/electroblots of various sources of O^6-MT: (A) recombinant enzyme, (B) HT29 cells, (C) VA13 cells, (D) normal liver, (E) lymphocytes from the same patient as D.

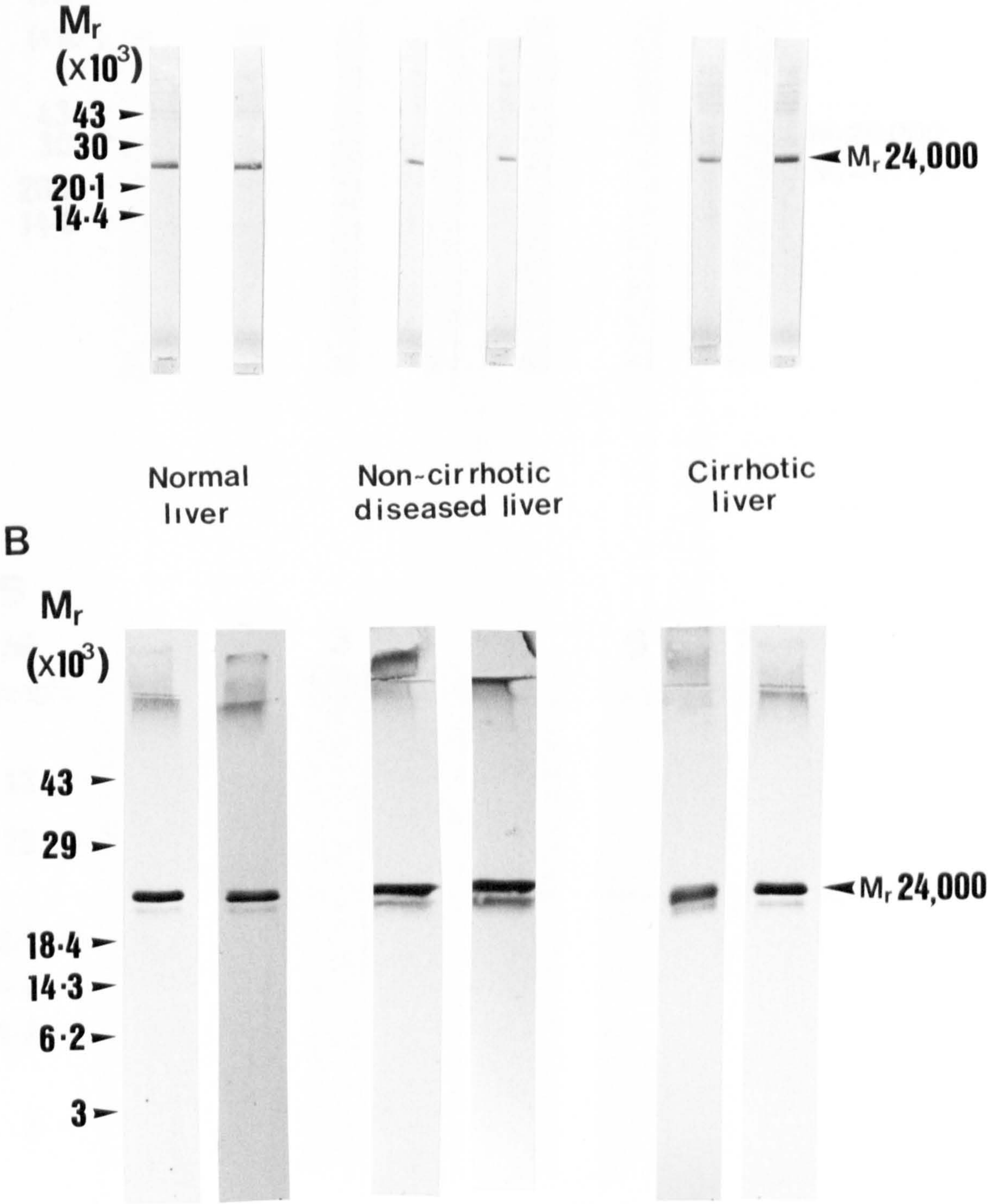


Figure 5.3.3a: SDS-PAGE/electroblotting of [^{3}H-Me] liver extracts prepared from indicated patient groups, with enzyme in corresponding blot strips detected by either (A) immunoprobing with C-terminal directed antibody (3µg/ml) or (B) fluorography.

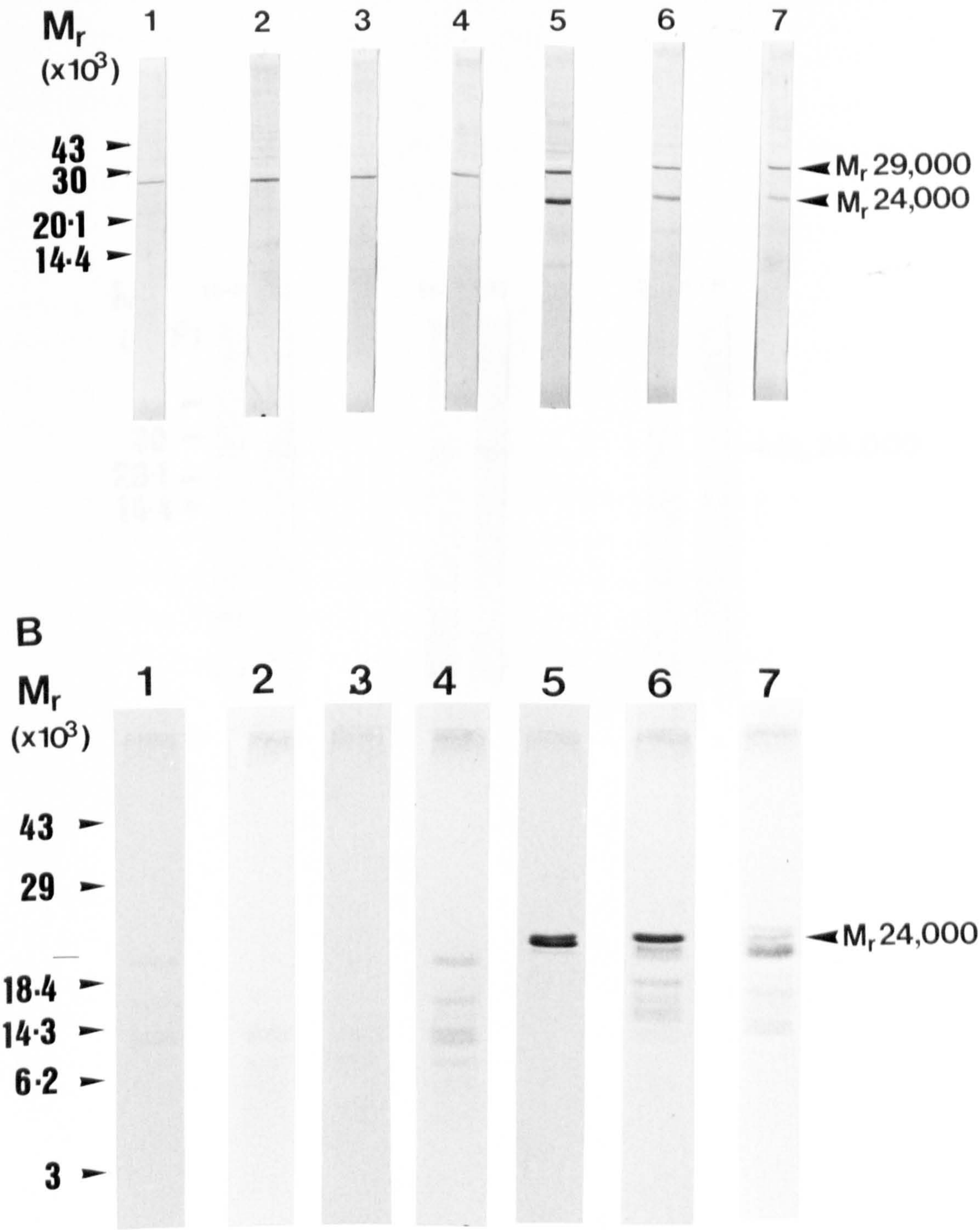


Figure 5.3.3b: SDS-PAGE/electroblotting of [^{3}H-Me] lymphocyte extracts prepared from 7 patients (lane 1-7), with enzyme in corresponding blot strips detected by either (A) immunoprobing with C-terminal directed antibody (3µg/ml) or (B) fluorography.

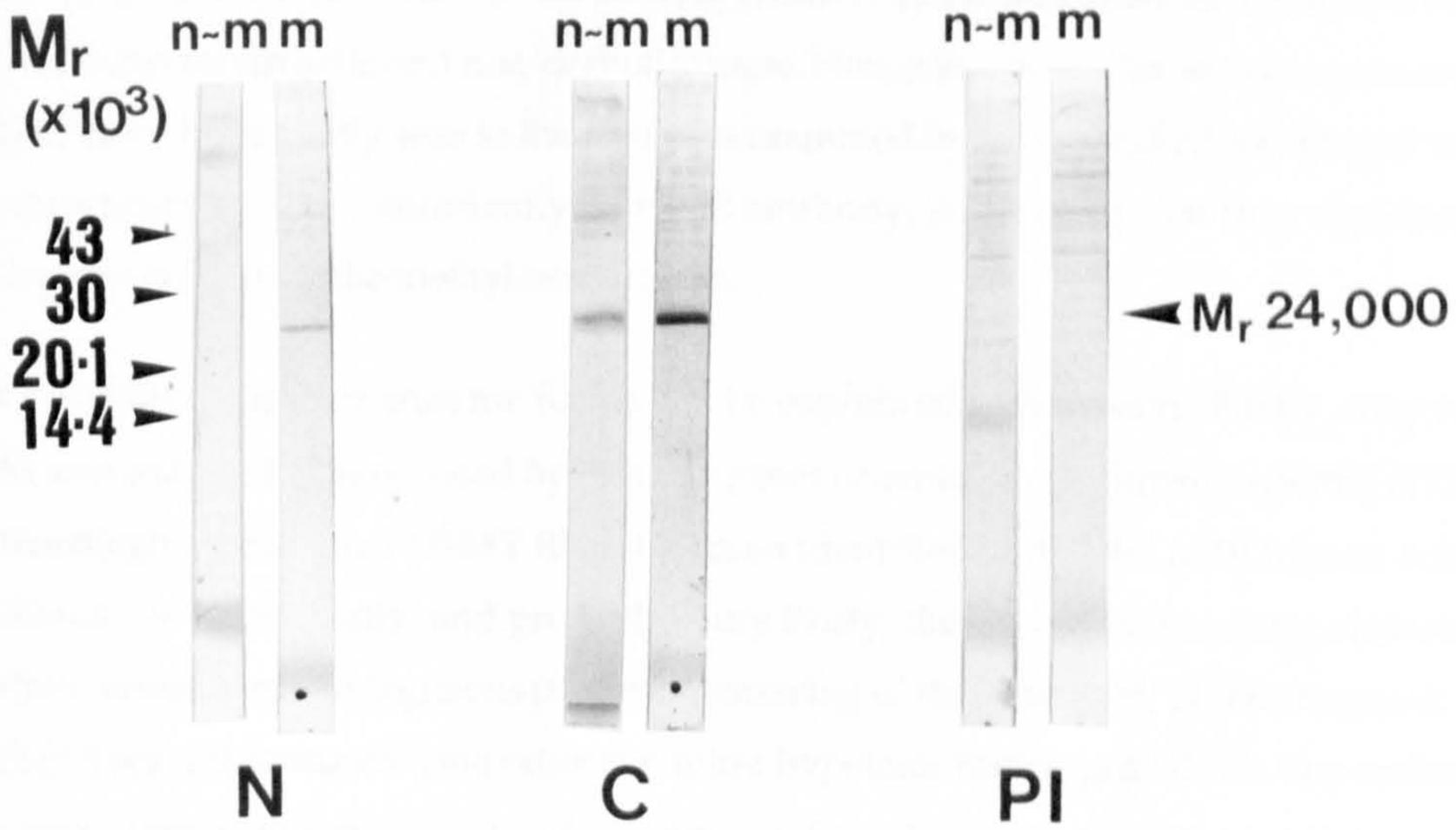


Figure 5.3.4: Detection of O^6-MT in SDS-PAGE/electroblots of normal liver extracts using N- and C-terminal directed antibodies (N and C respectively, used at 3µg/ml). Preimmune serum (PI), as IgG purified by protein A-Sepharose used at 6µg/ml, is shown for comparison. The liver extracts contain O^6-MT (25fmol per lane) which is in its native/non-methylated (n-m) form or methylated (m).

direct enzyme assay findings of low levels of native O^6-MT in cirrhosis. Analysis of extracts containing [^{3}H-Me] O^6-MT by SDS-PAGE/fluorography also allowed physicochemical analysis of the enzyme in cirrhotic liver tissue in an attempt to explain the low enzyme levels.

In *E.coli*, two distinct molecular weight forms of O^6-MT exist which have different functional characteristics, and are encoded by two different genes. The *ada* gene product has a M_r of 39,000 and levels increase following exposure to methylating agents whereas the *ogt* gene product has a M_r 19,000 and levels are not inducible. In this study, no similar higher molecular weight forms of O^6-MT, larger than M_r 24,000, were observed in human liver which might have explained the differences in enzyme levels between cirrhotic and non-cirrhotic tissue. However, molecular weight forms smaller than M_r 24,000 were consistently seen in liver extracts examined by SDS-PAGE/fluorography which were not recognised by the C-terminally directed antibody, suggesting that they represent C-terminally truncated forms of the methylated enzyme.

The presence of these smaller enzyme forms can be explained in two ways. Firstly, they could represent distinct enzyme forms encoded by different genes or arising from alternate splicing of O^6-MT mRNA. Interestingly, a truncated O^6-MT RNA has been identified in the HeLaMR human cell line (Fritz and Kaina, 1992). Secondly, and probably more likely, they could be products of protease digestion either arising from endogenous protease processing of the normal M_r 24,000 enzyme *in vivo*, or as a result of freeze/thawing enzyme extraction into a hypotonic buffer, procedures that are known to cause release of lysosomal proteases and consequent degradation of otherwise intact proteins, *in vitro*. The observed loss of up to 40% of O^6-MT activity in liver extracts upon storage (Chapter 3) supports a process of endogenous proteolytic degradation occurring over this time as it is expected that cleavage of an enzyme would affect activity. The enzyme in lymphocytes, however, appears more stable as shown by the maintenance of activity during prolonged storage. This contrasts with the more extensive enzyme cleavage that was seen when the lymphocyte extracts were examined by SDS-PAGE/fluorography, compared to liver extracts. These conflicting findings could be explained if C-terminal degradation was not associated with loss of enzyme activity. Indeed, recombinant protein which has been C-terminally truncated by 22 amino acids resulting in a protein of M_r19,700, similar to the smallest molecular weight form seen in liver in this study, retains functional activity (Elder et al, 1992). Interestingly, it seems that even N-terminal truncated O^6-MT, which has been reported for the yeast enzyme, also retains enzyme activity Xiao and Samson, 1992). The existence of both N and C-terminally truncated O^6-MT taken together with the genetic diversity seen in the N and C-terminal across species (Potter et al, 1991) suggests that these regions are not essential for functional enzyme activity .

The C-terminally truncated enzyme forms seen in this study were detected as [^{3}H-Me]O^6-MT in liver extracts by SDS-PAGE/fluorography. They may, therefore, represent native O^6-MT which had been degraded *in vivo*, prior to *in vitro* methylation. Alternatively, *in vitro* methylation may have triggered C-terminal truncation (Figure 5.4). The trigger for protease degradation may be a conformational change in the enzyme on methylation. A conformational change in O^6-MT has been

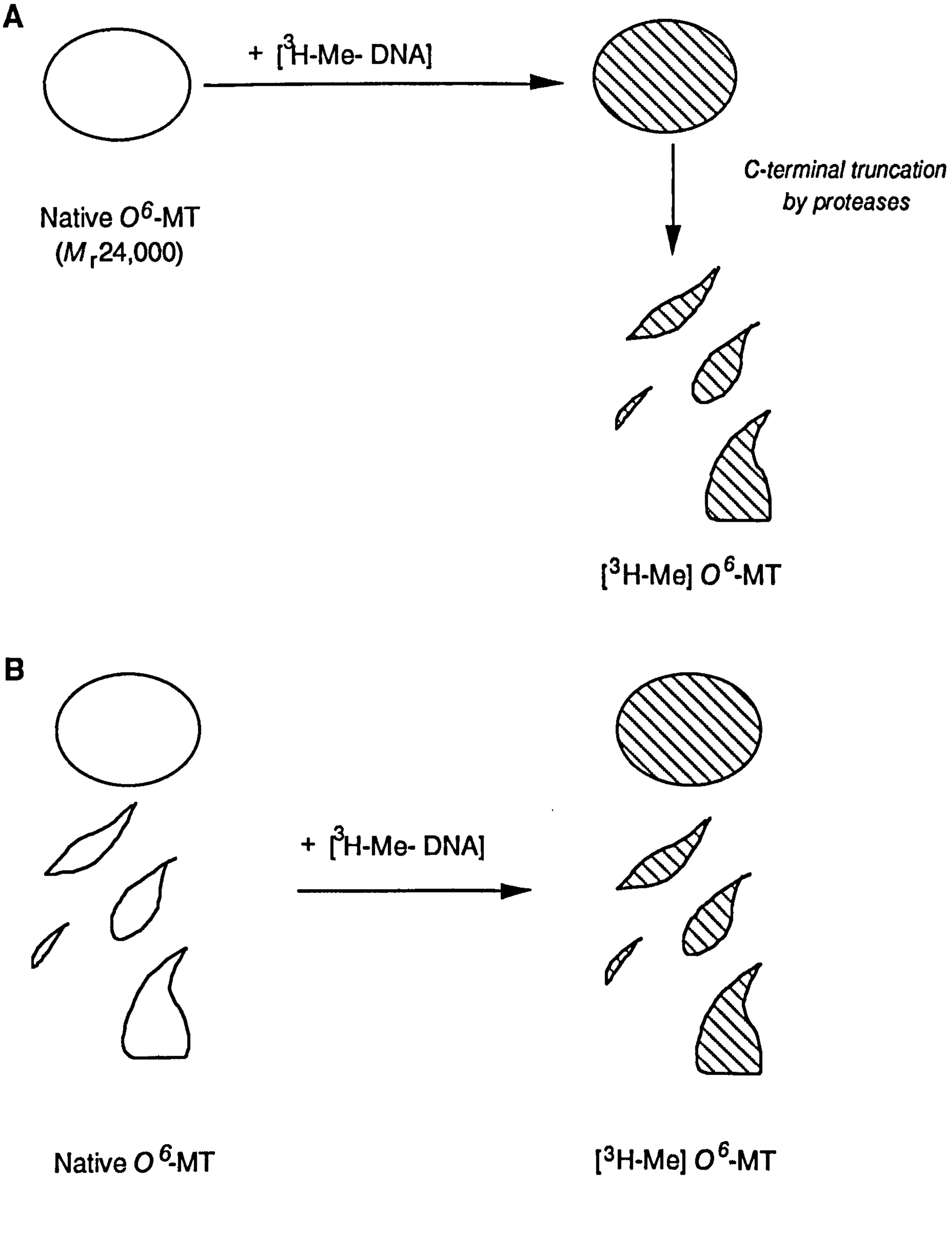


Figure 5.4: Hypotheses to explain the presence of C-terminal truncated O^6-MT in liver extracts. (A) Methylation of the native enzyme in vitro triggers protease degradation , or (B) native O^6-MT is truncated by endogenous proteases in vivo.

suggested to occur in the *E coli* protein on alkylation (Lindahl et al, 1988) and in this study the inability of the N-terminal directed antibody to recognise both native and methylated enzyme suggests that a conformational change might also occurs upon methylation of O^6-MT in human liver.

It is unknown how the inactive methylated protein is degraded *in vivo*, although several studies have tried to answer this question. Pegg et al (1991b) has suggested that in HT29 cells O^6-MT methylation is followed by rapid total enzyme degradation as opposed to progressive C-terminal truncation. Rapid enzyme degradation does not appear to occur in all cell types as Zhukovskaya et al, (1992) have detected endogenously methylated enzyme in cell extracts. They also showed differences in the ratio of native to methylated enzyme between different cell lines suggesting that the rate of enzyme degradation varies between cells. The findings of different C-terminally truncated enzyme forms in liver and lymphocytes in this study suggests that the pattern of degradation is also tissue-specific but not disease-specific as similar C-terminal truncated forms were seen in cirrhotic and non-cirrhotic liver. An attempt to discover whether the rate of enzyme degradation differed between cirrhotic and non-cirrhotic liver by measuring the ratio of native to endogenously methylated O^6-MT was unsuccessful because of the difficulty in quantitating the enzyme in immunoblots.

This study has confirmed the finding by direct enzyme assay (Chapter 3) of low levels of O^6-MT in association with cirrhosis. Furthermore, it has demonstrated that these low levels are not a reflection of either the differential expression of multiple molecular weight forms or the presence of smaller C-terminal truncated enzyme forms which have lost activity. Understanding the mechanisms underlying the low expression of O^6-MT in cirrhosis will depend on further characterisation of factors controlling enzyme synthesis and degradation *in vivo*.

5.5 Summary

DNA Repair By O^6-MT in Cirrhotic Liver

- O^6-MT levels (mean±SEM) as determined by SDS-PAGE/fluorography/densitometry

Normal liver (n=4)	853± 121 IOD/mg protein
Non-cirrhotic diseased liver (n=17)	781±71 IOD/mg protein
Cirrhotic liver (n=15)	393±54 IOD/mg protein

- O^6-MT levels were significantly lower in cirrhotic liver than non-cirrhotic diseased liver (p=0.0002) or normal liver (p=0.0015)

Physicochemical analysis of O^6-MT

- Conformational change in O^6-MT appears to occur in liver tissue following methylation

- Multiple molecular weight forms of O^6-MT

  - No molecular weight forms of O^6-MT higher than M_r 24,000 were identified in liver extracts.

  - Several lower molecular weight forms at M_r 23,200, 21,800 and 20,900, representing C-terminally truncated enzyme, were present in normal, non-cirrhotic and cirrhotic liver

  - More extensive C-terminal enzyme degradation was present in lymphocyte extracts compared to liver tissue.

Chapter 6

Expression Of Mutant p53 In Hepatocellular Carcinoma

6.1 Introduction

Persistence of the promutagenic DNA base lesion O^6-methylguanine, due to low levels of the repair enzyme O^6-MT, is known to lead to G to A transition mutations. Such mutations are commonly found within the p53 tumour suppressor gene in a wide range of human tumours. Thus, the process whereby HCC develops in a cirrhotic liver may involve the occurrence of mutations within the p53 gene because of failure to repair O^6-methylguanine .

The p53 tumour suppressor gene is located on the short arm of chromosome 17 (reviewed by Levine et al, 1991). It encodes a 53kd nuclear phosphoprotein which is expressed at low levels in normal cells which has the ability to complex with viral and cellular antigens (Lane and Crawford, 1979; Kern et al, 1991). Its precise function remains to be fully elucidated but it is likely to be involved in controlling cell proliferation as levels of the protein are influenced by the cell cycle and microinjection of antibodies to p53 prevent cells entering S phase (Reich and Levine, 1984; Mercer et al, 1982). Initial reports suggested that p53 levels were elevated in many human tumour derived cell lines in contrast to normal cells and that co-transfection of p53 with the *ras* oncogene resulted in cellular transformation (Lane and Crawford, 1979; Jenkins et al, 1988). This led to p53 being initially considered as an oncogene product. However, subsequent reports showed that the p53 clone used in these initial studies was mutant (Hinds et al, 1989). Moreover, wild type p53 was shown to be capable of suppressing cell proliferation and transformation (Finlay et al. 1989). Thus, p53 is now considered to act as a tumour suppressor gene with its involvement in cellular transformation being mediated either by loss of wild- type suppressor activity or by gaining dominant transforming activity through a mutation (Lane and Benchimol, 1990). Mutant p53 can transform cells in culture in the presence of low levels of wild type p53 (Hinds et al, 1989). This appears to be through its ability to bind to and inhibit the native protein as well as stimulating cell growth in the absence of wild type p53.

The genetic basis for mutant protein has been widely investigated and as a result, mutations of the p53 gene have been found to be a common alteration in human cancers (reviewed by Hollstein et al, 1991). Mutations are usually accompanied by chromosome 17 losses involving the p53 locus on the other allele which reinforces the tumour suppressor function of wild type p53 (Nigro et al, 1989). p53 mutations occur in both familial cancer syndromes (Li-Fraumeni) and sporadic cancer (Srivastava et al, 1990; Malkin et al , 1990). Mutations are usually found within four highly conserved regions of the gene, namely exons 5 to 8. The exact position and nature of the p53 base changes are characteristic of tumour type. In human lung and liver G : C to T : A transversions predominate whereas G : C to A : T transitions are commoner in colon, oesophageal and brain tumours. Mutations are spread across exons 5 to 8 in oesophageal and lung tumours but tend to occur in CpG dinucleotide "hotspots" in colorectal and brain tumours. These differences are likely to reflect exposure to different carcinogens.

Abnormalities of the p53 gene in HCC were first observed in cell lines. In one study, five out of seven lines had abnormalities of protein expression (Bressac et al, 1990). Subsequent sequencing of the p53 gene in HCC revealed specific mutations at codon 249 in 50% of HCC from China and south Africa, both areas of high aflatoxin exposure (Bressac et al, 1991; Hsu et al, 1991). These mutations were all G to T transversions, known to be a possible consequence of aflatoxin exposure (Muench et al, 1983). In support of a role for aflatoxin in causing this mutation was the demonstration, in a global study of p53 mutations in HCC, of a correlation between aflatoxin exposure and G to T mutations specifically at codon 249 (Ozturk et al, 1992). Larger studies analysing p53 mutations from different geographical areas have now been completed (Table 6.1). These studies have shown that in an area such as Britain, with low exposure to aflatoxin and Hepatitis B infection, p53 mutations are less common; when they do occur they are neither exclusively confined to codon 249 nor G to T transversions. Although the frequency of p53 mutations is high in areas where HCC is prevalent not all the mutations are specific codon 249 transversions suggesting that other carcinogens may be important in the aetiology of HCC in these areas. A significant number of p53 mutations are G to A, the type of DNA base damage expected from O^6-methylguanine.

Chromosome 17 allele loss occurs frequently in HCC (Nishidia, et al 1992) and often accompanies p53 mutations (Nishida et al, 1993). Point mutations do occur without allele loss and allele loss has also been demonstrated in the absence of p53 mutations raising the possibility of other yet unrecognised tumour suppressor genes on the short arm of chromosome 17 that may also be important in the pathogenesis of HCC (Scorsone et al, 1992).

The biological significance of these various point mutations however is far from clear. Not all point mutations have the same functional effect on p53 and this includes the inability of some mutations to stabilise the protein (Malkin et al, 1990). As it is the mutant protein that ultimately effects cell regulation and thus the malignant phenotype it is important that the presence of mutant protein is assessed together with genetic alterations in the p53 gene.

Although a study, from the North East Of England, has shown that only 10% of HCC had p53 gene mutations this was only a small study involving 19 patients (Challen et al, 1992b). The aim of this study was to use immunohistochemistry to examine a much larger series of hepatocellular carcinoma for the presence of mutant p53 protein.

6.2 Patients and methods

6.2.1 Clinical material

Formalin-fixed paraffin-embedded archival material was available for study from 55 cases of hepatocellular carcinoma seen at the Freeman Hospital. These patients formed part of the retrospective clinical study of HCC reported in Chapter 2. The mean age of the patients was 70 (range 43-84) with a male : female ratio of 2:1. 77% of patients had biopsy proven cirrhosis and

Table 6.1: Geographical differences in prevalence and type of p53 mutations.

Country	Patient Number	Patients with p53 mutations	p53 G to T mutations	p53 G to A mutations	Aflatoxin exposure	HB sAg +ve patients	Codon 249 mutations	Reference
China	36	58%	NA	NA	High	100%	100%	Scorsone et al, 1992
China	16	50%	90%	0%	High	NA	100%	Hsu et al, 1991
S . Africa	10	50%	80%	0%	High	80%	100%	Bressac et al, 1991
Tiawan	61	33%	30%	10%	High	67%	NA	Sheu et al, 1992
Japan	53	32%	38%	8%	Low	13%	0%	Nishida et al, 1993
Japan	169	29%	18%	16%	Low	45%	14%	Oda et al, 1992a
Tiawan	20	15%	0%	0%	High	85%	0%	Hosono et al, 1993
Germany	13	15%	0%	0%	Low	30%	0%	Kress et al, 1992
England	19	10%	0%	50%	Low	10%	0%	Challen et al, 1992b

NA - data not available

serological markers of Hepatitis B infection were present in 15 (27%) cases, 4 patients had HBsAg and in 11 cases HBcAb alone was present.

6.2.2 Immunostaining

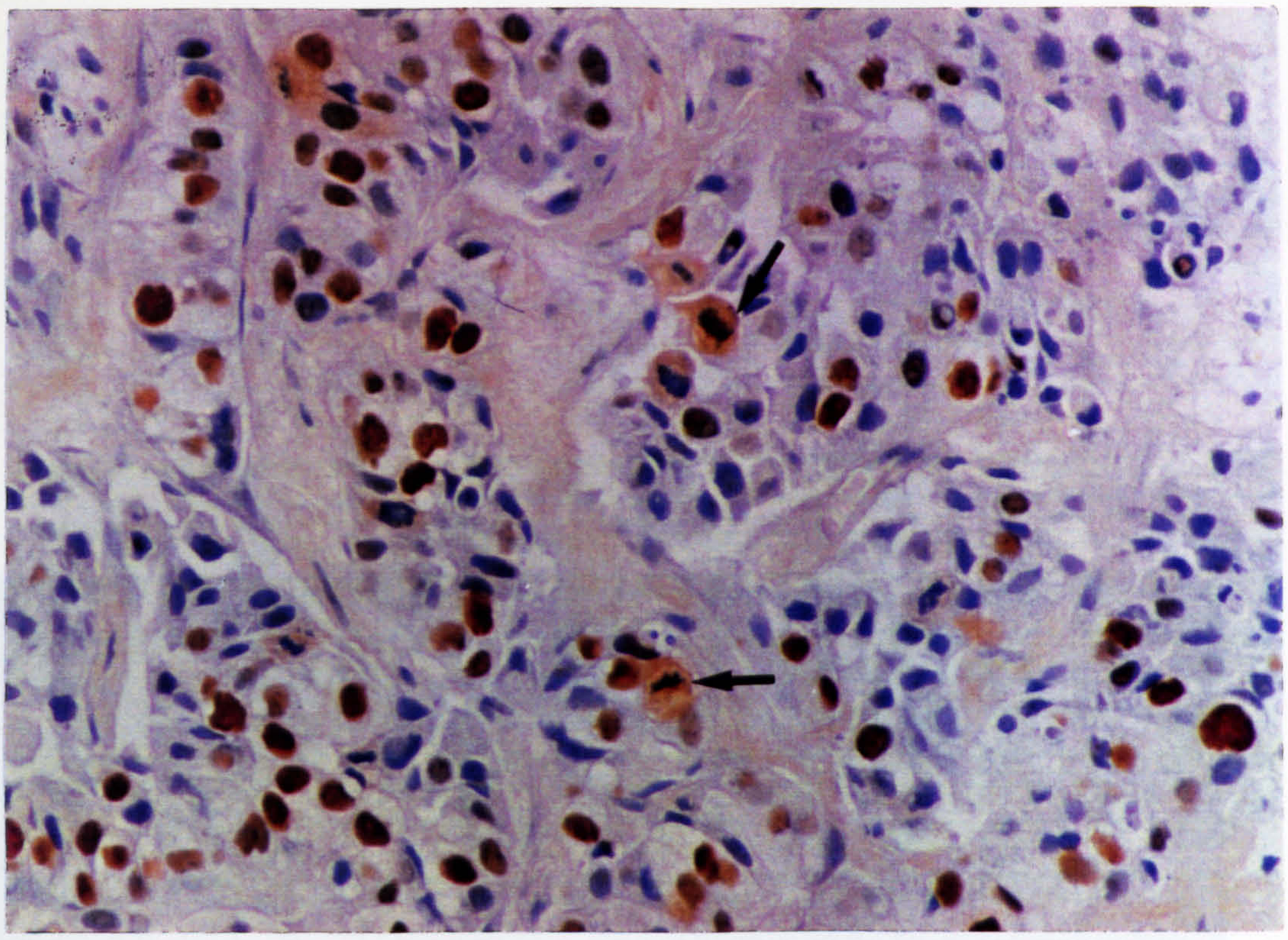
3μm sections were cut from paraffin blocks and immunostained as described in Chapter 4 with the following modifications. Endogenous peroxidase was blocked by incubation in 0.5% hydrogen peroxide in methanol for 10 minutes and the tissue was then equilibrated in TBS. Non-specific antibody binding was blocked by pre-incubation of the sections with either 3% normal swine serum (NSS) or 3% normal rabbit serum (NRS), depending on whether the primary antibody was polyclonal or monoclonal respectively, for 10 minutes. p 53 was identified using two primary antibodies raised against recombinant p53 protein, CM-1 and DO-7. CM-1 is a rabbit polyclonal antibody, raised against human wild type p53 produced in a bacterial expression system using a full length p53 cDNA, and used at a dilution of 1/1000 (Bartek et al, 1991). The monoclonal antibody DO-7, used at a dilution of 1/100, is also raised against recombinant human wild type p53 and has shown to be specific for the denaturation-resistant epitope at the N-terminus of the protein (Vojtesek et al, 1992). Incubation with 100μl per section of primary antibody was performed overnight at 4° C in a moist incubation tray. The sections were then washed in two 5 minute exchanges of TBS. Immunostaining was detected using an avidin-peroxidase system. The tissue was incubated with 100μl of 1/1000 biotinylated swine anti-rabbit immunoglobulin (Dako) diluted in NSS or 100μl of 1/500 biotinylated rabbit anti-mouse immunoglobulin (Dako) in NRS for 30 minutes at room temperature, for sections stained with CM-1 or DO-7 respectively. Following two further 5 minute washes in TBS 100μl of Strep ABC complex (1μl streptavidin, 1μl biotinylated peroxidase, 100μl TBS)(Dako) was placed on each section for a further 30 minutes. Immunoreactivity was then detected, after washes in TBS, using 3'3 diaminobenzidine as described in chapter 4. Sections were counterstained with Mayers haematoxylin and mounted in DPX.

A malignant fibrohistiosarcoma of the liver, shown to have a G to A mutation at codon 248 of the p53 gene, was used as a positive control . Negative controls consisted of replacement of the primary antibody by normal swine or rabbit immunoglobulin.

6.3 Results

Using the monoclonal antibody DO-7 immunolabelling was detected in 5/55 (9%) tumours (Figure 6.3a). The staining was focal, involving less than 10% of the malignant cells, but of uniform intensity and was confined to the nucleus except in those cells undergoing mitosis. Immunostaining with the polyclonal antibody CM-1 was also only seen in the 4 DO-7 positive tumours with a similar focal nuclear distribution. However background staining was higher using CM-1 compared to DO-7 (Figure 6.3a). In the 5 positive HCC cases surrounding cirrhotic tissue was present in only one and was negative for p53 (Figure 6.3b). In 10 p53-negative tumours, surrounding cirrhotic tissue was also negative.

A



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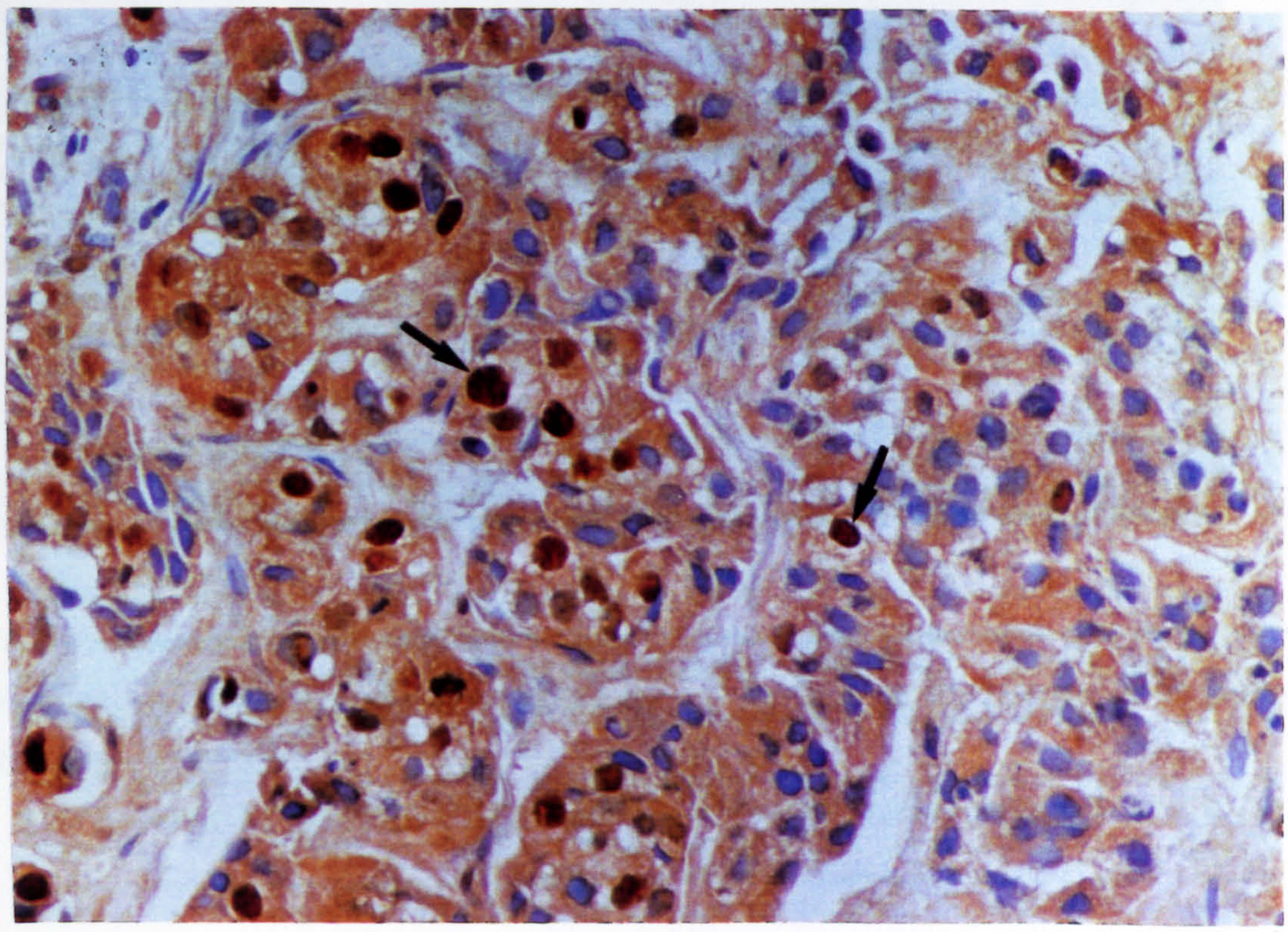

Figure 6.3a: Expression of mutant p53 in hepatocellular carcinoma. (A) Intense staining with monoclonal antibody DO-7 within nuclei of a well differentiated hepatocellular carcinoma. Cytoplasmic staining is seen in those hepatocytes undergoing mitosis (arrowed). (B) Positive nuclear staining (arrowed) with anti-p53 polyclonal antibody CM-1 in malignant cells. There is greater background cytoplasmic immunoreactivity with this antibody compared to DO-7.

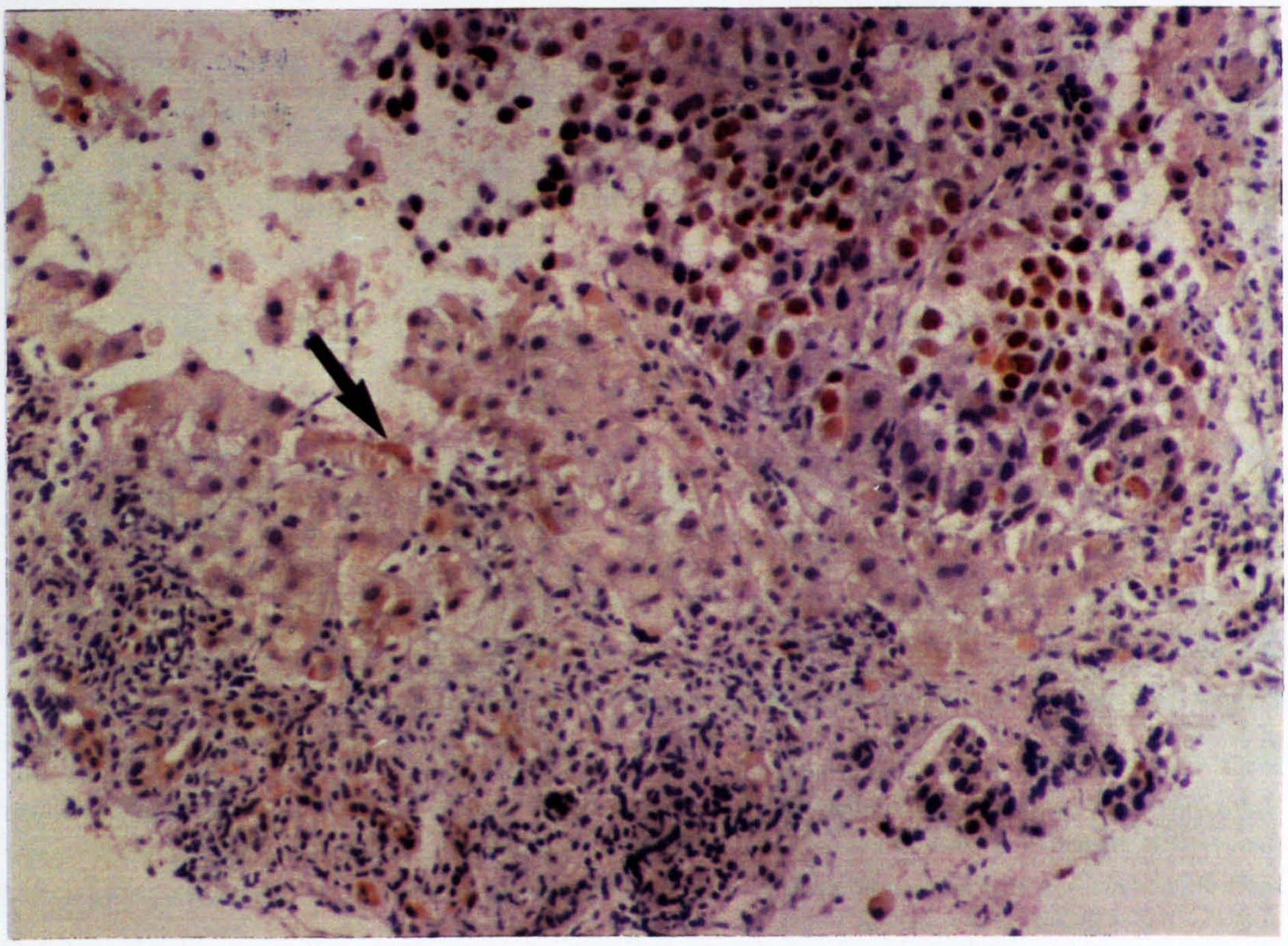

Figure 6.3b: Expression of mutant p53 in malignant hepatocytes as demonstrated by nuclear immunoreactivity with monoclonal antibody DO-7 but not in hepatocytes of adjacent cirrhotic tissue (arrowed).

The clinical features of the 4 cases expressing mutant p53 is shown in Table 6.3a. The median survival of these cases was 6 weeks (range 1-60) which compares to a median survival of 10 weeks (range 1-96) for those patients whose tumours did not express mutant p53.

6.4 Discussion

Although the frequency of p53 gene mutations in hepatocellular carcinoma varies geographically, from 10% in low incidence areas to 50% in high incidence areas (reviewed in Figure 6.1a), it is still unknown whether this variation is also reflected in differences in expression of mutant protein and cell growth. Native (wild type) p53 protein can not normally be detected immunohistochemically because of its short half life (Gannon et al, 1990; Oren et al, 1981). Many p53 gene mutations result in expression of protein with a prolonged half life and resulting cellular accumulation (Finlay et al, 1988). The ability to detect p53 expression immunohistochemically thus usually occurs when the protein is mutant, but also has been shown to sometimes reflect stabilization of native protein by interaction with cellular and viral (eg SV40) proteins (Lane, 1979; Werness et al, 1990; Lehman et al, 1991). More recently abnormal expression of wildtype p53 protein has been found in normal cells of a cancer family patient, implying impaired degradation (Barnes et al, 1992).

In this study expression of p53 was demonstrated in 9% of British tumours. Other studies have shown there is overall a good correlation between p53 gene mutations and detectable protein immunohistochemically (Bartek et al, 1990; Davidoff et al, 1991; Rodrigues et al, 1990; Iggo et al, 1990). In a previous British study in our laboratory of 19 HCC, two (16%) had p53 gene mutations, one of which was detected immunohistochemically using the polyclonal antibody CM-1 (Challen et al, 1992b). Combining these two studies (10 patients were included in both studies) 5 out of 64 (8%) of British HCC express mutant p53 protein.

Not only was expression of mutant protein uncommon in this series but in those cases where it did occur the distribution was focal. Levels of native p53 protein are known to vary throughout the cell cycle , accumulating during G_1 and falling following cell division (Bischoff et al, 1990). Mutant p53 protein may also be regulated by the cell cycle and the focal distribution may reflect this. A heterogeneous pattern of p53 gene mutations has also been demonstrated in nodules from multiple HCC in Japanese patients, suggesting a multifocal rather than clonal origin for the tumour in the majority of patients (Oda et al, 1992b). The absence of staining in cirrhotic tissue adjacent to tumour expressing mutant p53 is consistent with a role for the protein in the late events of hepatocarcinogenesis.

It is of interest that in gastric cancer expression of p53 mutant protein has been considered to be a poor prognostic indicator (Martin et al, 1992). In this retrospective study it was not possible to evaluate the prognostic significance of p53 expression in hepatocellular carcinoma but preliminary evidence suggests that it may contribute to an aggressive tumour phenotype (Laurent-Puig et al,1992). A prospective study is now necessary to investigate the value of p53 expression as a prognostic indicator.

Table 6.3a: Clinical features of patients with HCC expressing mutant p53.

Age	60	76	77	44
Sex	M	M	M	F
Aetiology	Cryptogenic cirrhosis	No cirrhosis	Cryptogenic cirrhosis	HBV-related cirrhosis
HBs Ag	Negative	Negative	Negative	Positive
αFP (IU/l)	<10	670	90000	92000
Survival (weeks)	60	1	6	unknown

This study has demonstrated the feasibility of detecting mutant p53 in paraffin-embedded material. Immunoreactivity with the novel reagent DO-7 was comparable to that seen with CM-1 but with the advantage of significantly less background staining. A large prospective analysis of p53 expression with DO-7 is therefore possible using routinely processed paraffin-embedded biopsy material rather than frozen tissue (Laurent-Puig et al, 1992).

The low frequency of p53 gene mutations seen in British HCC is reflected in a similar frequency of mutant protein expression. These findings when taken with p53 data from other areas of the world indicates that there are marked geographical difference in aberrant p53 expression in HCC. This suggests differences in the molecular mechanisms of hepatocarcinogenesis with p53 being less important in low incidence areas such as Britain.

The results of this study also indicate that p53 is unlikely to be involved in the process of hepatocarcinogenesis through the mechanism of DNA damage occuring in cirrhotic tissue because of failure to repair O^6-methylguanine. The possibility that G to A mutations occuring in other oncogenes and tumour suppressor genes play a role in the development of hepatocellular carcinoma in cirrhosis requires further evaluation.

6.5 Summary

Expression Of Mutant p53

•Detected immunohistochemically using two antibodies raised against recombinant p53 (polyclonal CM-1 and monoclonal DO-7) with comparable focal, nuclear staining.

• HCC (n = 55)	• 5/55 (9%) positive
•Cirrhotic tissue surrounding HCC (n=11)	• 0/11 (0%) positive

Chapter 7

Expression Of C-*erb* B-2 Oncoprotein In Hepatocellular Carcinoma

7.1 Introduction

C-*erb* B-2 is a proto-oncogene located on chromosome 17 (Fukushige et al, 1986) which encodes a transmembrane phosphoglycoprotein of M_r 190,000 (Yamamoto et al, 1986). The protein consists of an external domain linked to an internal domain by a transmembrane region, and has a 50% homology with the epidermal growth factor receptor (EGFR). Its homology with EGFR has led it to be considered as a growth factor receptor although its ligand is still unknown.

Overexpression of the c-*erb* B-2 protein has been reported in many malignant cell lines (Gullick et al, 1987) and human tumours, such as breast (Slamon et al, 1987), bladder (Wright et al, 1991) and lung (Roncalli et al, 1991). Protein overexpression correlates with gene amplification (Venter et al, 1987) which appears to be one mechanism whereby the c-*erb* B-2 proto-oncogene is activated. *Neu*, the rat equivalent of c-*erb* B-2, by contrast is activated by point mutations (Bargmann et al, 1986). A direct role for C-*erb* B-2 in tumorigenesis is suggested by the finding that expression of activated c-*neu* in transgenic mice induces malignant transformation of mammary epithelium (Muller et al, 1988). Moreover, exposure of *neu* transformed cell lines to monoclonal antibodies, raised to the external domain of the protein, results in loss of the transformed phenotype suggesting an additional role for the protein in maintaining the transformed phenotype (Drebin et al, 1986). C-*erb* B-2 overexpression has been associated with a poor prognosis in breast carcinoma (Dykins et al, 1991). The observation that tumour growth in rats can be inhibited *in vivo* by the use of monoclonal antibodies directed against the external domain of neu has raised the possibility of using immunotherapy targetted to the c-*erb* B-2 protein in human tumours (Drebin et al, 1988).

A preliminary study recently suggested that 80% of HCC overexpress c-*erb* B-2 oncoprotein as demonstrated immunohistochemically using a polyclonal antibody (Voravud et al, 1989). This is much higher than that reported in other tumours, such as breast cancer where the incidence is 20-30% (Corbett et al, 1990) and suggests a role for this protein in human hepatocarcinogenesis. It also raises the possibility of using immunotherapy against the c-*erb* B-2 oncoprotein in the treatment of HCC which might improve the prognosis of what is presently a relatively chemotherapeutic resistant tumour.

The aim of this study was therefore to analyse the expression of c-*erb* B-2 hepatocellular carcinoma patients in the North East of England using a highly specific monoclonal antibody to detect the protein immunohistochemically.

7.2 Patients and methods

Archival formalin-fixed paraffin-embedded liver biopsy material from 26 cases of HCC was available for study; sufficient material only remaining in 26 of the 55 cases examined for mutant p53 in Chapter 6. Clinical information was obtained on all patients from medical records. All 26 patients were European Caucasian with the exception of 1 patient with HCC who was Japanese. The age

range of the patients was 54-73 with a male to female ratio of 19 to 7. Four patients had serological markers of hepatitis B infection (HBsAg) and 23 patients has serum α- fetoprotein levels greater than 500IU/l (normal <10).

Immunohistochemistry was performed as described in Chapter 4 . Briefly 3μm sections were cut from tissue blocks. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 minutes. The primary monoclonal antibody NCL-CB11 was raised against a synthetic peptide corresponding to the C-terminus of the internal domain of the c-*erb* B-2 protein (Corbett et al, 1990). Sections were incubated overnight at 4°C with a 1 in 40 dilution of NCL-CB11. Immunostaining was then performed using an indirect immunoperoxidase method. The secondary antibody was a peroxidase-conjugated mouse ant-rabbit antibody (Dako) used at a dilution of 1 in 20. A positive control (a case of breast carcinoma known to over express c-*erb* B-2) was included in all experiments.

7.3 Results

The presence of cell membrane labelling was considered an essential criterion for identification of c-*erb* B-2 overexpression; this pattern is demonstrated by the positive breast control in Figure 7.3a. Only 2 out of 26 HCCs had membrane staining and in both cases the immunoreactivity was focal. One of these was a clear cell variant of HCC; the intensity of the membrane staining was much weaker than in the breast control (Figure 7.3a). The trabecular tumour which over expressed c-*erb* B-2 had also stained for mutant p53 in Chapter 6. Weak cytoplasmic staining was also seen in 5 out of 26 HCCs (Figure 7.3b).

7.4 Discussion

The c-*erb* B-2 oncoprotein is a transmembrane receptor and positive immunohistochemical staining in the form of membrane immunoreactivity has been shown to correlate with overexpression of the protein and gene amplification in breast cancer. In this study, only 2 out of 26 hepatocellular carcinoma showed such positive membrane staining using a recently developed monoclonal antibody to the c-*erb* B-2 protein NCL-CB11. These results contrast markedly with those reported by Voravud *et al* (1989) who found that 12/14 (86%) of hepatocellular carcinoma stained positively with the polyclonal antibody, 21N to the c-*erb* B-2 oncoprotein. However, they did not distinguish between cytoplasmic and membrane staining when reporting their findings. This is important as the significance of cytoplasmic staining remains one of conjecture; cytoplasmic staining in the absence of membrane staining does not correlate with overexpression of c-*erb* B-2.

Cytoplasmic staining has been reported with both the polyclonal antibody 21N and the monoclonal antibody NCL-CB11 (Corbett et al, 1990). In this study, cytoplasmic immunoreactivity was detected in 5/26 (19%) of tumours. Possible explanations for cytoplasmic staining include crossreactivity with an unknown protein, as both antibodies react with a band of M_r 150,000 on Western blotting (Corbett et al, 1990; Gullick et al, 1987), abnormal processing of the RNA and protein, or internalisation of the c-*erb* B-2 receptor. Internalisation of the epidermal growth factor receptor, which has homology

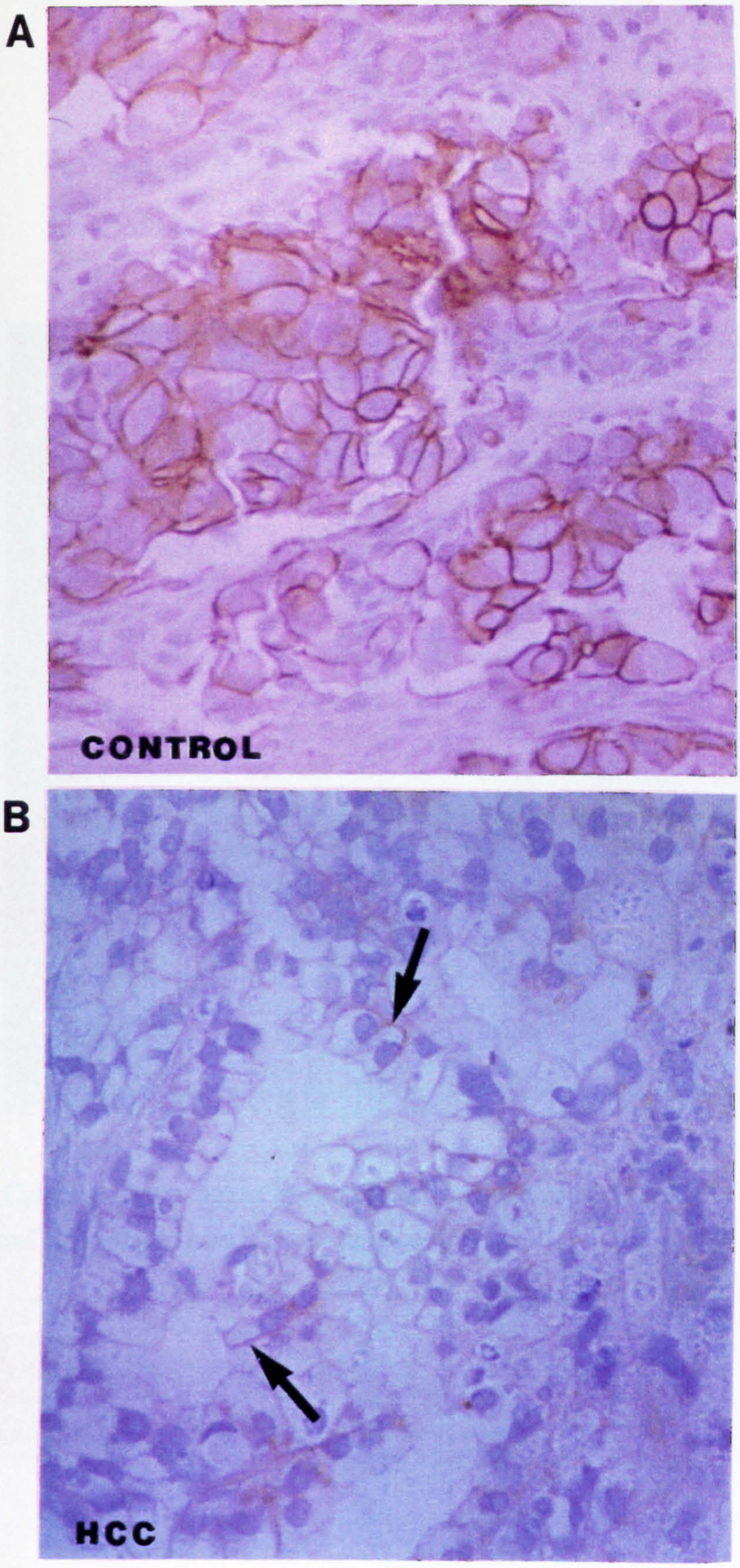

Figure 7.3a: (A) Membrane immunoreactivity with c-erbB-2 antibody NCL-CB11 in a positive control section of breast carcinoma. (B) Membrane staining (arrowed) in a clear cell variant of hepatocellular carcinoma with NCL-CB11.

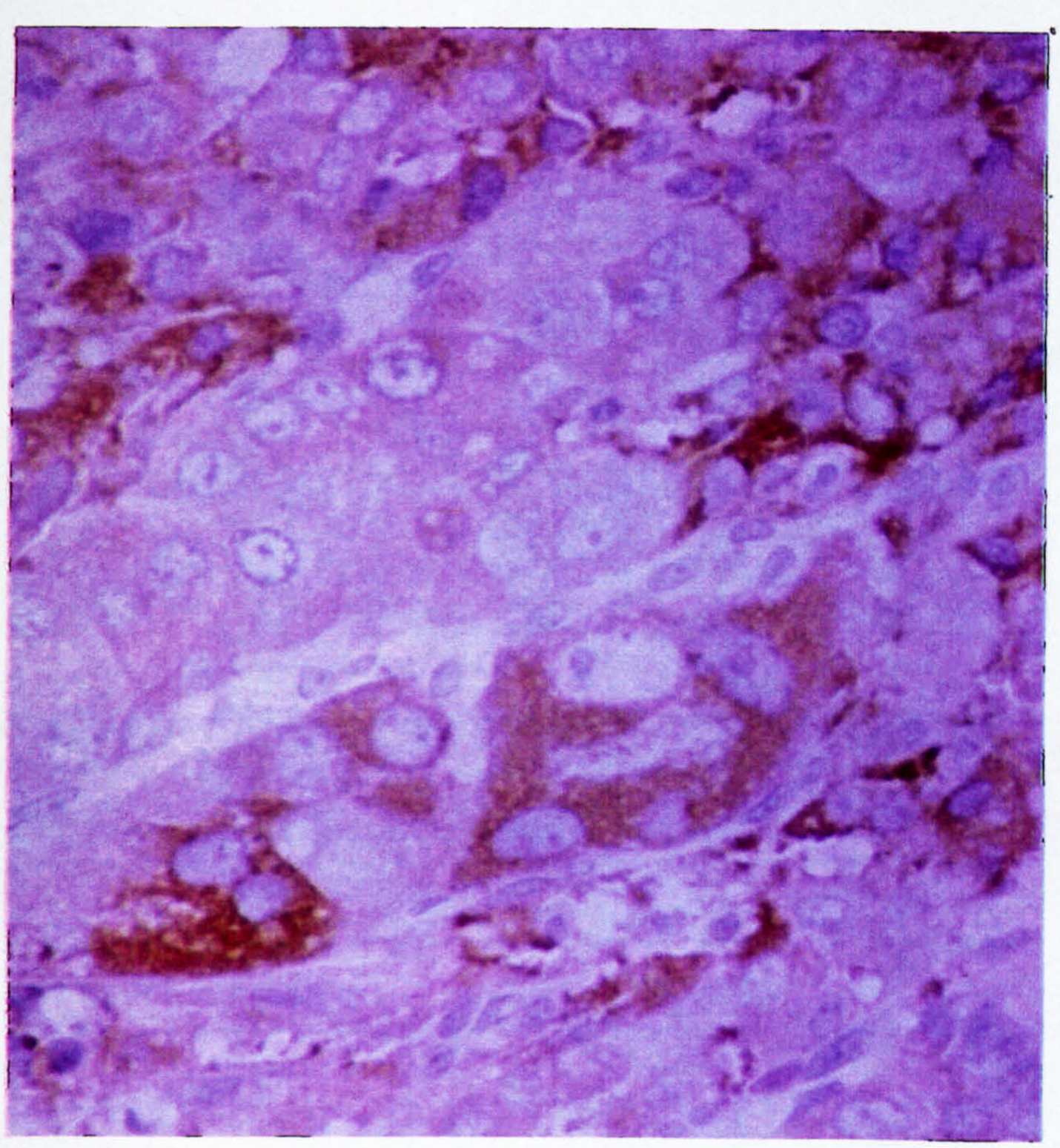

Figure 7.3b: Cytoplasmic immunoreactivity with the c-erbB-2 antibody NCL-CB11 in a moderately well differentiated hepatocellular carcinoma.

with the c-*erb* B-2 protein, has been demonstrated in epidermoid carcinoma (A431) cells (Miller et al, 1986). In that study EGFR was demonstrated on both the cell surface and endosomal compartments in a transfer pathway of EGF/EGFR complex to the lysosomes. C-*erb* B-2 has also been demonstrated immunohistochemically in endoplasmic reticulum using electron microscopy (Miller et al, 1986). In the 2 HCCs which showed cytoplasmic staining in this study, no c-*erb* B-2 RNA was detected confirming that overexpression of c-*erb* B-2 oncogene, comparable to that found in breast adenocarcinoma was not occuring (Collier et al, 1992).

The results thus indicate that overexpression of the c-*erb* B-2 proto-oncoprotein is uncommon in hepatocellular carcinoma and is therfore unlikely to be important in hepatocarcinogenesis. Moreover immunotherapy using the c-*erb* B-2 transmembrane receptor cannot be considered as potential therapy in these tumours.

7.5 Summary

C-*erb* B-2 Oncoprotein Expression in HCC

- 2/26 (8%) membrane immunoreactivity
- 5/26 (19%) cytoplasmic immunoreactivity

Overexpression of c-*erb* B-2 oncoprotein is uncommon in British HCC

Chapter 8

Expression Of Transforming Growth Factor Alpha In Hepatocellular Carcinoma.

8.1 Introduction

Transforming growth factor alpha (TGF-α) is a 50 amino acid mitogenic secretory polypeptide which has 30% homology with the 53 amino acid epidermal growth factor (reviewed by Derynck , 1988). It binds to the epidermal growth factor receptor (EGFR) (Todaro et al, 1980; Massague, 1983) and thus activates receptor tyrosine kinase activity leading to autophosphorylation, a prerequisite for stimulating DNA synthesis (Reynolds et al, 1981).

The TGF-α gene, located on chromosome 2, was cloned in 1984 and found to encode a 160 amino acid precursor polypeptide (Derynck et al, 1984). This precursor consists of a 100 amino acid extracellular domain that includes the N -terminal signal sequence and the 50 amino acid mature peptide, a hydrophobic transmembrane domain and 35 amino acid cytoplasmic domain. This transmembrane precursor is proteolytically cleaved at the cell surface resulting in secretion of mature TGF-α. Both the mature TGF-α and the transmembrane precursor are biologically active (Brachmann et al, 1989; Wong et al, 1989)

TGF-α was first isolated from the culture medium of retrovirally transformed fibroblasts and subsequently from other transformed cells but not the parental cell lines suggesting a role in transformation (De Larco et al, 1978; Kaplan et al, 1980).This was supported by transfection studies in which rat fibroblasts transfected with human TGF-α cDNA lost anchorage-independant growth and formed TGF-α secreting tumours when injected into nude mice (Rosenthal et al, 1986). In man, TGF-α RNA has been detected in a range of malignant cell lines and tumours (Derynck et al, 1987).

In the liver TGF-α mRNA production increases in rat hepatocytes (but not non-parenchymal cells) following partial hepatectomy and is temporally related to increased DNA synthesis and rising EGFR mRNA. This suggests that TGF-α acts as a mediator of liver regeneration through an autocrine loop (Mead and Fausto, 1989). Identification of immunoreactive TGF-α in proliferating hepatocytes of rat liver following carbon tetrachloride induced damage lends further support to this hypothesis (Burr et al, 1993).

Several pieces of evidence suggest that TGF-α may be important in hepatocarcinogenesis. First, a number of HCC cell lines, including the human HCC cell line HepG2, synthesize and secrete TGF-α (Derynck et al, 1987; Liu et al, 1988; Luetteke et al, 1988). Second, in transgenic mice human TGF-α overexpression results in the development of multifocal hepatocellular carcinoma (Jhappan et al, 1990). Finally, high urinary levels of TGF-α have been detected in patients with hepatocellular carcinoma(HCC) (Chuang et al, 1991; Yeh et al, 1987).

The aims of this study were (a) to immunolocalise TGF-α in normal human liver, (b) to identify the expression of TGF-α in human HCC indicated by the presence of mature peptide using immunohistochemistry, (c) to compare TGF-α immunoreactivity in tumour and cirrhotic tissue from individual patients.

8.2 Materials and methods

8.2.1 Case Material

10% buffered formalin-fixed paraffin-embedded archival material from 28 cases of hepatocellular carcinoma were studied; the presence or absence of mutant p53 and c-*erb*B-2 oncoprotein had been established immunohistochemically in these cases in the previous chapters. In 20 cases, tumours had developed on a background of established cirrhosis, non-tumour tissue was available for study in 13 of these. Four cases of normal human liver was also included for immunohistochemical analysis. Full clinical details were obtained from medical records 27 patients were European Caucasian, the male to female ratio was 1.8:1, and 19 patients had an α fetoprotein level of >500µg/l. Serological markers of Hepatitis B virus infection were present in 9 patients, 4 had HBsAg and 5 with HBcAb alone.

8.2.2 Immunohistochemistry.

3µm sections were used for immunostaining, as described in Chapter 4 with the following modifications. Following hydration through graded alcohols endogenous peroxidase was blocked with 0.5% hydrogen peroxide in methanol for 10 minutes and the sections were preincubated with 0.1% trypsin for a further 10 minutes. The primary antibodies used were a polyclonal antibody 26T (gift of Dr W Gullick, ICRF, Hammersmith Hospital, London) used at a dilution of 1 in 20 (Barton et al, 1991) and a monoclonal antibody Ab-2 (Oncogene Science) diluted to 1 in 20 (Sorvillo et al, 1990). All incubations with primary antibody were performed at 4°C overnight. Immunoreactivity with the polyclonal antibody 26T was detected using an indirect immunoperoxidase method as described in Chapter 4. Staining with Ab-2 was detected using an avidin-biotin system (ABC) with biotinylated rabbit anti-mouse immunoglobulin antibody as the secondary antibody as described in Chapter 6. 3'3 Diaminobenzidine was used as the chromagen in all experiments. Pre-incubation of primary antibody with cognate peptide and omission of the primary antibody acted as negative controls. Sections of duodenal mucosa known to express TGF-α were used as a positive control.

8.3 Results

8.3.1 Normal liver

Using the antibody Ab-2, TGF-α immunoreactivity was seen in very occasional hepatocytes with strong cytoplamic staining in bile duct epithelium. Labelling was absent from perisinusoidal cells (lipocytes), Kupffer cells and sinusoidal endothelial cells. Intense staining was also seen in bile duct epithelium stained with 26T but in a membrane rather than a cytoplasmic distribution.

8.3.2 Tumours

4/28 cases of HCC showed membrane immunoreactivity with 26T (Figure 8.3.2). There was associated cytoplasmic staining in 3 cases but this was absent from a single case of clear cell variant of HCC. A

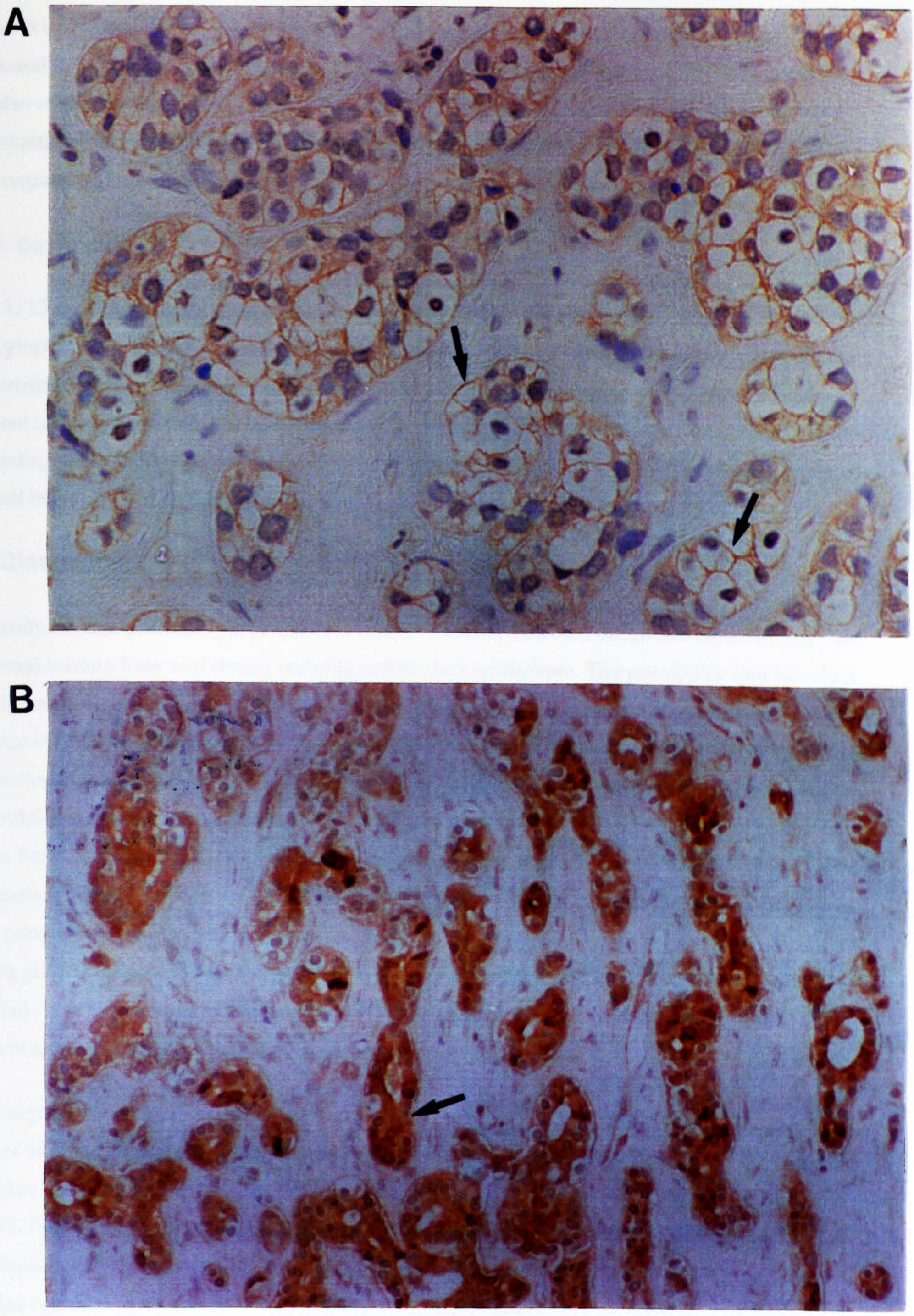

Figure 8.3.2: TGF-α expression in hepatocellular carcinoma. (A) Membrane immunoreactivity (arrowed) in a clear cell variant of hepatocellular carcinoma with the polyclonal antibody 26T. (B) Cytoplasmic immunolabelling within the same tumour with the monoclonal antibody Ab-2 (arrowed).

further 8 tumours had cytoplasmic staining, but no membrane immunoreactivity, although this was weak and in 4 cases was only focal. The 12 cases which had shown positive immunoreactivity with 26T also stained with Ab-2 but staining was confined to the cytoplasm with no apparent membrane accentuation(Figure 8.3.2). No Ab-2 positive 26T-negative tumours were identified. Pre-incubation with cognate peptide abolished TGF-α membrane and cytoplasmic immunoreactivity.

8.3.3 Cirrhosis

Only 1/13 cases of cirrhosis showed immunoreactivity, within hepatocytes, with anti TGF-α and this was cytoplasmic with both 26T and Ab-2 antibodies (Figure 8.3.3). In this TGF-α positive case HBsAg was present whereas the other 12 cases of cirrhosis examined were not hepatitis B virus related .The adjacent tumour from this case had shown similar staining limited to the cytoplasm. In 4/12 of the remaining cases TGF-α expression was limited to the tumour. Immunostaining was also seen in residual bile ducts and proliferating ductules in cirrhotic cases (Figure 8.3.3).

8.4 Discussion

This study has demonstrated the presence of immunoreactive TGF-α in occasional parenchymal cells of normal human liver and strong staining in bile duct epithelium. The possibility that bile duct epithelium exists as a source of TGF-α has not been previously considered but it is consistent with the presence of TGF-α mRNA and protein in other normal human glandular epithelial tissues such as the gastrointestinal tract (Malden et al, 1989). No detectable TGF-α was found in perisinusoidal cells, sinusoidal endothelial cells or Kupffer cells. It is of interest that fixed tissue macrophages in other tissues have been shown to express the peptide (Rappolee et al, 1988). Although lack of TGF-α immunoreactivity in Kupffer cells may reflect functional heterogeneity of macrophages in growth factor production, the possibility that these findings are a reflection of rapid secretion of TGF-α from hepatic macrophages (Kupffer cells), resulting in undetectable cellular protein, cannot be excluded. In previous studies, macrophage TGF-α production was detected using sensitive RNA detection methods rather than analysis of immunoreactive protein.

TGF-α expression, as demonstrated by membrane and cytoplasmic immunoreactivity, was seen in 8/28 (28%) of HCCs. The pattern of staining probably reflects the synthetic pathway of TGF-α as the antibodies used react with both the mature protein and its precursor (Barton et al, 1991; Hazarika et al, 1987). The 160 amino acid precursor, containing the mature 50 amino acid TGF-α, is translated in the endoplasmic reticulum where it is anchored to the membrane by its C-terminus. Mature peptide is then cleaved during transport to the plasma membrane where it is secreted and available for membrane interaction (Teixido et al, 1987; Bringman et al,1987). Cytoplasmic immunoreactivity therefore represents either precursor or mature TGF-α whereas membrane staining suggests either persistent membrane bound precursor or secreted TGF-α bound to its receptor (EGFR).

Membrane staining was not seen with the monoclonal anti-TGF-α antibody Ab-2 and is likely that this reflects binding to a different region of TGF-α from 26T as Ab-2 was raised against the 50 amino

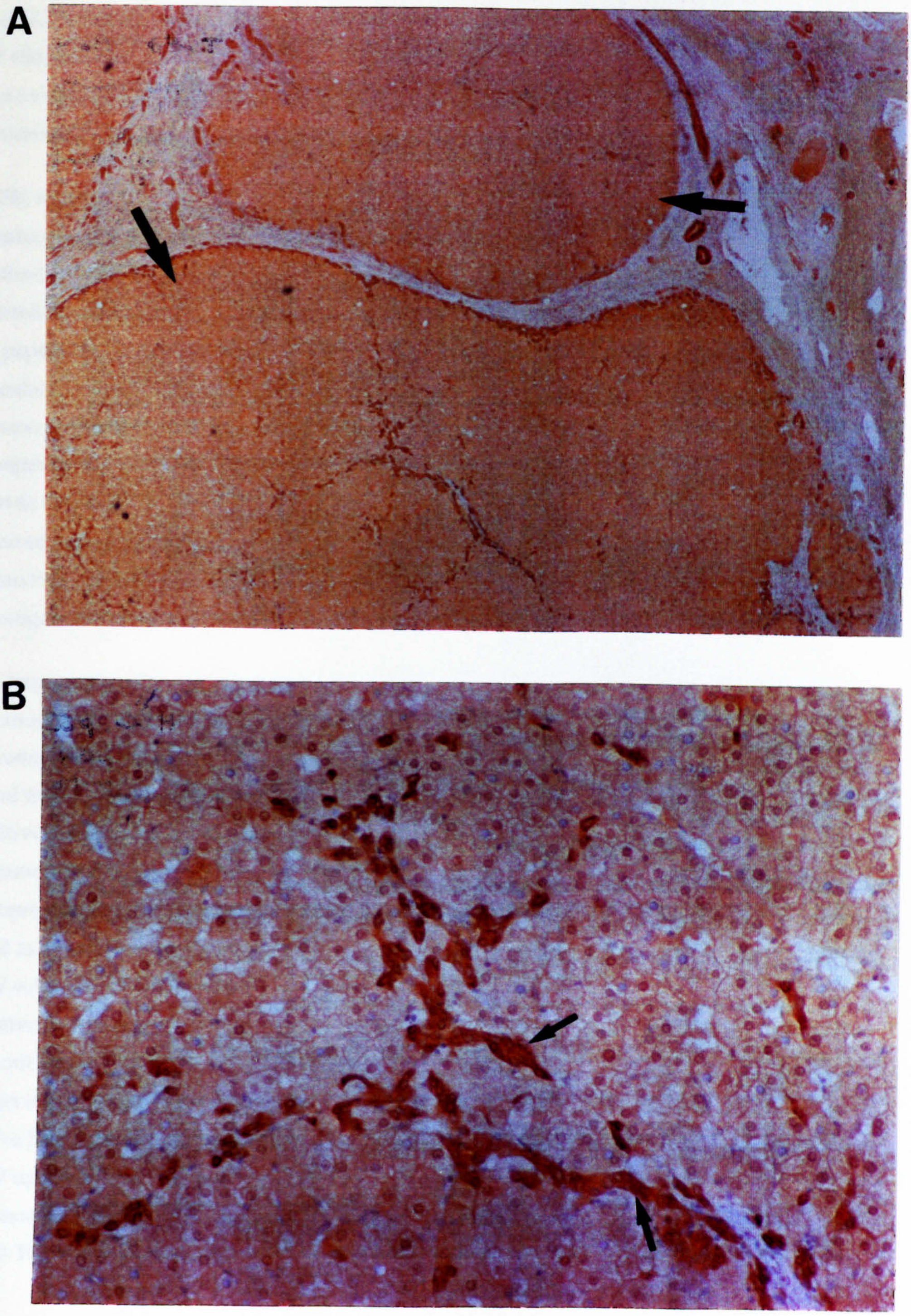

Figure 8.3.3: TGF-α expression in cirrhosis. (A) Cytoplasmic staining with Ab-2 within hepatocytes (arrowed) in a case of hepatitis B related cirrhosis. (B) Cirrhotic tissue at a higher magnification illustrating the associated strong immunoreactivity within residual bile ducts and proliferating ductules (arrowed).

acid TGF-α peptide and not the 17 amino acid C-terminus used to produce the polyclonal antibody 26T (Barton et al, 1991; Sorvillo et al, 1990). However, synthesis of TGF-α by these malignant hepatocytes is supported by the detection of specific RNA from 2 tumours which exhibited TGF-α immunoreactivity (Collier et al, 1993).

EGFR, required to complete an autocrine loop of TGF-α stimulation, is known to be present on hepatocytes (Dunn et al, 1986). However *in vitro* studies to document the effect of TGF-α or EGFR antibodies on hepatocellular carcinoma cell growth, in order to verify this hypothesis , are still awaited. In addition to the postulated autocrine role of TGF-α in hepatocarcinogenesis expression of the peptide by malignant cells may exert paracrine effects on surrounding mesenchymal cells. The vascularity of HCC may be partly explained by paracrine effects of TGF-α which is known *in vitro* to cause vasodilatation by a direct action on vascular epithelium (Gan et al, 1987). Paracrine effects of hepatocyte and bile duct-derived TGF-α on perisinusoidal cells (lipocytes), which are important in hepatic fibrogenesis, is also suggested by the finding that TGF-α stimulates the proliferation of cultured perisinusoidal cells *in vitro* (Bachem et al, 1989). HCC subtypes characterised by excess extracellular matrix, such as sclerosing and fibrolamellar variants, may be rich in TGF-α ; studies to investigate this are now indicated.

The importance of studying non-tumour tissue when investigating the role of growth factors in carcinogenesis has been emphasized by a recent study of TGF-α in colorectal cancer as tumour expression mirrored that in surrounding normal tissue (Malden et al, 1989). However, differences were noted in our study when HCC was compared with that of surrounding cirrhotic tissue. In 5 TGF-α positive tumours in which surrounding cirrhotic tissue was present, only one case showed immunoreactivity within parenchymal cells of cirrhotic nodules. These results contrast with those of a recent study from China where over 80% of cirrhotic tissue from patients with HCC stained with Ab-2 as did the equivalent number of tumours (Hsia et al, 1992). However, all these patients had HBV-related cirrhosis in contrast to the cirrhotic group in this study in which only one was HBsAg positive. It is of note, however, that this was the only case expressing TGF-α in both cirrhotic and tumour tissue. The presence of immunoreactive TGF-α in HBV-related cirrhosis and tumours, may reflect impaired peptide secretion due to coexistant viral infection, as colocalisation of HBsAg and TGF-α has been observed in individual cells (Hsia et al, 1992). An alternative explanation is that HBV upregulates TGF-α expression which is supported by the observation that TGF-α RNA levels increase following transfection of the hepatoblastoma cell line, Hep G2, with HBV (Tabor et al, 1992). TGF-α upregulation may therefore be invoved in the pathogenesis of HBV-related HCC.

8.5 Summary

Expression Of Transforming Growth Factor alpha

Normal liver	• expression confined to bile duct epithelium.
HCC	• 8/28 (28%) expressed TGF-α as indicated by cytoplasmic immunoreactivity (Ab-2) and membrane and/or cytoplamic staining with 26T.
Cirrhosis	• cirrhotic tissue surrouding HCC positive tumours only expressed TGF-α in 1/7 cases. This tumour was the only HBs Ag tumour examined.
	• no TGF-α expression was seen in tissue surrounding HCC negative cases.

Chapter 9

Final Discussion

The following discussion will focus on two issues; firstly, the significance of the finding of low levels of the DNA repair enzyme O^6-MT in cirrhotic tissue with respect to the risk of developing HCC; secondly, on the role of oncogenes, tumour suppressor genes and growth factors on hepatocarcinognesis in Britain, and in comparison to other geographical areas.

O^6-MT levels, measured using the direct enzyme assay, described in Chapter 2, were significantly lower in cirrhotic tissue compared to non-cirrhotic diseased liver and normal liver; the lack of correlation seen between diseased liver and lymphocyte levels indicated that this deficiency is tissue- and disease-specific. These enzyme assay findings were confirmed by SDS-PAGE of extracts followed by flurography and quantitiative densitometry where the native enzyme which had been methylated and radiolabelled was detected as an expected M_r 24,000 band. This suggests that cirrhosis may be a risk factor for HCC because of the persistence of the unrepaired DNA base lesion O^6-methylguanine, which in the presence of cell proliferation, a characteristic feature of cirrhosis, is known to produce G to A transition mutations, a known mechanism of oncogene activation. The risk of HCC developing in a cirrhotic liver is also dependant on advanced age, male sex and actiology of cirrhosis. In the context of the interindividual variation in enzyme levels in cirrhotic tissue it is tempting to speculate that these factors may also affect O^6-MT levels. A larger study of cirrhotic liver tissue is now required to fully establish the relationship between these other risk factors and O^6-MT levels. The relative contribution of environmental and hereditary factors to liver enzyme levels is unknown. It might be possible to assess any genetic component if it could be shown that lymphocyte levels correlated with levels in normal liver, so allowing the latter to be used as a surrogate marker for normal tissue levels. This would allow O^6-MT levels to be measured in a large group of cirrhotic and non-cirrhotic patients and their families.

The disadvantage of assessing O^6-MT levels in cirrhotic liver extracts is that it does not take into account differences in enzyme levels at the cellular level; namely between parenchymal and non-parenchymal cells, and between individual hepatocytes. It is more likely that the number of hepatocytes not expressing O^6-MT will be more important in determining the susceptibility of cirrhotic tissue to DNA base damage than the overall tissue levels. Unfortuantly the peptide antibodies raised to O^6-MT in this study were unable to immunolocalise the enzyme in tissue sections. However, O^6-MT has recently been demonstrated in normal liver by another group using antibodies raised against recombinant enzyme (Lee et al, 1992). In that study O^6-MT was localised to hepatocytes but the distribution was heterogenous. It is likely, but as yet unproven, that such heterogenous O^6-MT expression also occurs in cirrhotic liver. Sufficient amounts of recombinant enzyme are now available to allow polyclonal antibodies to be developed in an attempt to assess the distribution of O^6-MT in cirrhotic tissue; this work is currently in progress.

The tissue expression of O^6-MT can also, potentially, be evaluated using *in situ* hybridisation for mRNA. A successful method has been reported using a digoxygenin labelled oligonucleotide in human kidney (Wani et al, 1992). In preliminary work, which I have carried out, it has not been

possible to reproduce these findings in human liver (unpublished observations), and the finding of non-specific nuclear staining, similar to that reported by Wani et al, throws doubt on the specificity of their findings in human kidney. The use of multiple oligonucleotides or riboprobes, possibly radiolabelled with [^{35}S] rather than digoxygenin, may be needed to improve the sensitivity of *in situ* hybridisation for O^6-MT RNA.

The mechanism underlying the low levels of O^6-MT observed in cirrhosis remain one of conjecture as the factors controlling enzyme synthesis and the normal rate of cell turnover are still unknown. The presence of C-terminal truncated enzyme has been previously reported in human liver. Although this was confirmed in the present study in both cirrhotic and non-cirrhotic tissue this enzyme form appears to retain enzymatic activity, and cannot therefore account for the low levels seen in cirrhosis.

Although the finding of low levels of O^6-MT in cirrhosis suggests increased susceptibility to alkylating agent damage, and hence mutation and carcinogenesis it must be emphasised that the level of O^6-MT is only one factor determining a suseptibility of a tissue to environmental carcinogens. The other factors include the ability of the liver to metabolise alkylating agents to reactive metabolites, the extent to which DNA damage occurs as well as the extent of DNA proliferation at the time of adduct formation. Ideally all four factors need to be assessed in cirrhotic tissue simutaneously before an assessment of an individuals risk of developing HCC can be made. Another complicating factor is that carcinogenesis is a dynamic process with the sequence of events leading to HCC occuring over a prolonged time period. Ideally O^6-MT needs to be measured on serial liver biopsies, from normal through cirrhosis to the time of tumour development. Levels of O^6-MT have been measured in HCC and peritumour liver tissue in a study of 21 patients from Japan (Isowa et al, 1991) but there was little difference in enzyme levels between tumour and surrounding liver tissue. As these tumours appeared to have occured on the basis of a non-cirrhotic liver it would be interesting to know whether the levels in the peritumour tissue differed from enzyme levels in cirrhotics uncomplicated by HCC and cirrhotic tissue surrounding HCC in Japanese patients.

The contribution made by G to A mutations, produced by O^6-alkylguanine, to hepatocarcinogenesis is dependant on these mutations occurring within the coding region of proto-oncogenes or tumour suppressor genes involved in cell proliferation and differentiation. The sequence of events in terms of oncogene activation, altered tumour suppressor gene function and the role of peptide growth factors, which occur during tumorigenesis are unresolved. A genetic model has been proposed for colon cancer where the availability of preneoplastic tissue has allowed the sequence of molecular events to be evaluated (Fearon and Vogelstein, 1990). The salient features of this model are: firstly, mutational activation of oncogenes and inactivation of tumour suppressor genes both occur; secondly, mutations in at least four to five genes are required for tumour production; and thirdly, the total accumulation rather than the order of molecular events is responsible for determining the tumour's biological properties. The difficulty in applying this model to HCC is the lack of a recognised early stage of malignant transformation. However, as in colon cancer multiple pathways appear to occur. In HCC

these different pathways appear to be geographically determined, probably related to different aetiological factors. This is demonstrated in Table 9 which shows that whilst expression of mutant p53, c-*erb* B-2 oncogene product and TGF-α are uncommon in Britain, compared to HCC from high incidence areas, the opposite is seen with *ras* mutations. The high incidence of codon specific p53 mutations in high incidence areas has been explained by an epidemiological association with aflatoxin exposure. However, interestingly, site-specific p53 mutations do not occur in aflatoxin-B_1 induced rat hepatic hyperplastic nodules (Hulla et al, 1993). The presence of p53 mutations in tumours from areas of low aflatoxin but high hepatitis B exposure suggests the latter may prove to have a role in producing these mutations. Certainly TGF-α expression appears to be up regulated by hepatitis B infection and is probably involved in the pathogenesis of HBV- related HCC.

We are still far from fully understanding the mechanisms involved in human hepatocarcinogenesis. This work has, however, suggested the existence of different pathways probably determined by the involvement of different aetiological agents. Thus as further oncogenes and tumour suppressor genes are recognised their expression must be assessed in HCC from both high and low incidence areas. The role of alkylating agents and other environmental carcinogens deserve further study in HCC from Britain in the context of the lack of an association with a recognised aetiological agent in most tumours. As cirrhosis is a universal risk factor worldwide the finding of deficient DNA repair by O^6-MT in British cirrhotics needs to be evaluated in other geographical areas. The possibility that O^6-MT levels could be used as a marker to indicate an increased risk of a cirrhotic patient developing HCC and thus a need for closer screening is an interesting concept. The results of a prospective follow up study of the 48 patients studied to asertain if enzyme levels correlate with the development of HCC is now needed in an attempt to answer this question.

Table 9: Geographical differences in expression of oncogenes, tumour suppressor genes and growth factors in hepatocellular carcinoma.

Oncogene/growth factor	% Abnormal Expression	
	Low incidence area (Britain)	High Incidence area
ras	**24% *** (Challen et al, 1992a)	**0% *** (Tada et al, 1991)
p53	**9%** (Chapter 6)	**29 -58% *** (Table 6.1a)
***C-erb* B-2**	**8%** (Chapter 7)	**73%** (Voravud et al, 1990)
TGF-α	**28%** (Chapter 8)	**80%** (Hsia et al, 1992)

* gene mutations

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